Fate and Transport of Vertebrate DNA in Surface Water Environments:

Developing a Basis for Quantification through Environmental DNA Monitoring

By

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Submitted to the graduate degree program in Civil, Environmental, and Architectural Engineering and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Many environmental engineering applications require sampling of DNA to be effective. Organisms shed DNA into the environment, and that environmental DNA (eDNA) can be collected, extracted, amplified, and quantified to provide information about the shedding organisms. Regardless of the target organism, shed eDNA is subject to environmental degradation and partitioning into various compartments. Fate and transport of eDNA in aquatic systems was examined using two vertebrate organisms (invasive bigheaded carps, *Hypophthalmichthys* spp., and endangered Topeka shiners, *Notropis topeka*). Target eDNA concentrations were higher in sediment than the overlying water column and were correlated with biomass density of the target fish. Target eDNA in sediments was detectable and quantifiable at least 132 days after removal of the fish, suggesting eDNA persistence in aquatic systems may be significantly longer than previously reported. Degradation rates were highly correlated with initial eDNA concentrations in ponds with bioturbation and reduced macrophytes, suggesting degradation may be dominated by enzymatic hydrolysis in those systems.

N. topeka DNA from museum specimens was extracted and sequenced for cytochrome oxidase 1 (COI), cytochrome oxidase b (CytB), NAD dehydrogenase 2 (ND2), and the control region (D-loop). Such data are rare, and these tissue extractions and sequences represent important contributions to preservation of an endangered species. Both detection probability and concentration of *N. topeka* eDNA in water samples increased at biomass densities two to three times larger than naturally occurring schools (80 versus 20 to 30 fish). After an initial spike, fish eDNA in water dropped below detection limits within 7 days, regardless of stocking density. However, at 14 days detection in high density tanks increased to initial spike levels and remained so at 26 days after stocking. Fish eDNA was detected in water 27 days after fish removal, regardless of density, though at lower detection probability than during fish presence. Despite consistent detection, water column concentrations of fish eDNA did not exceed 20% of the initial spike over the course of 335 days. eDNA monitoring must account for partitioning and differential fate in the environment. Therefore, future studies should focus on models incorporating resuspension, ecological condition, and the mechanisms of degradation.

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TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

EXECUTIVE SUMMARY

Microbial research, pathogen source tracking, antibiotic resistance studies, bioremediation monitoring, biological conservation, invasive species tracking and other environmental engineering applications require environmental sampling to be effective. Organisms shed DNA into the environment, and that environmental DNA (eDNA) can be collected, extracted, amplified, and quantified to provide information about the shedding organisms. eDNA has been widely used in environmental engineering applications, but has only recently been adopted for observation and monitoring of vertebrates, especially for fish in aquatic systems. Monitoring of these organisms can be difficult, costly, and time consuming, especially for endangered species where minimal contact is best for preservation and for invasive species where maximum detection is necessary at low densities.

Regardless of the target organism, shed eDNA is subject to environmental degradation by ultraviolet light, chemical depurination, and enzymatic hydrolysis, and it partitions into various environmental compartments including open water, sediments, biofilms, and surface microlayers. Differential degradation rates have been observed in these different compartments, with the shortest residence time in open water. Sediment particles, especially clays, can reduce degradation of eDNA both by providing physical protection from enzymes and by directly sorbing enzymes themselves. Differential fate and transport of vertebrate eDNA in the environment are not well understood. Therefore, research is required to understand the implications of monitoring organisms using eDNA.

Using two vertebrate organisms (one a large-bodied invasive fish, *Hypophthalmichthys* spp., and the other a small-bodied endangered fish, *Notropis topeka*), fate and transport of eDNA in aquatic systems were examined. Transport of eDNA to sediments and degradation of eDNA studies were performed with *Hypophthalmichthys* spp., and novel genetic sequencing, quantitative PCR assay design, and a density-dependent eDNA concentration studies were performed with *N. topeka*. Since best practices for analysis of eDNA were rapidly evolving over the course of the project, care was taken to incorporate new discoveries to the greatest extent possible, including separation of high and low copy

extractions, separation of pre and post PCR processing, testing for inhibition, rigorous decontamination and internal process controls, minimization of freeze/thaw cycles of extracts, and use of low-bind plastics throughout sample processing.

Fish eDNA concentrations were higher in sediment than the overlying water column and were correlated with biomass density of the target fish (*Hypophthalmichthys* spp.), but resuspension of sediments and ecological changes associated with grass carp can cloud biomass effects. Internal positive controls for quantitative polymerase chain reaction (qPCR) assays can confirm lack of inhibition during amplification, and are recommended for every assay. Similarly, composites of sediment cores exhibited lower relative standard errors than individual cores and are therefore recommended for sediment eDNA analysis. Fish eDNA in sediments was detectable and quantifiable at least 132 days after removal of the fish, suggesting persistence in aquatic systems may be significantly longer than previously reported. Degradation of eDNA in sediment was modeled using a power function, and degradation rates differed across various ecological conditions and biomass loadings. Degradation rates were highly correlated with initial concentrations in ponds where grass carp were present in relatively high densities, suggesting degradation may be driven primarily by enzymatic hydrolysis in those systems.

Fish DNA was also extracted from museum archived and vouchered tissues for 185 individuals of N. topeka, and four mitochondrial regions were sequenced from a subset of the extracts: cytochrome oxidase 1 (COI), cytochrome oxidase b (CytB), NAD dehydrogenase 2 (ND2), and the non-encoding control region (D-loop). Since this fish is an endangered species and both tissue samples and reported DNA sequences are rare, these tissue extractions and sequences represent important contributions to the ongoing preservation of the species. Similar extractions and sequencing were carried out for 10 individuals of *Notropis stramineus* (the closest genetic relative of *N. topeka*) and individuals of 9 other co-occurring species. Using the gene sequences generated by this study and additional sequences from GenBank, species specific primers were developed for *N. topeka* using *N. stramineus* and co-occurring species as an outgroup. The primers were able to reliably amplify *N. topeka* DNA from tissue extracts at

various concentrations, in mixtures with non-target tissue extracted DNA at various concentrations, and in both pure water and water collected from ponds and tanks at the University of Kansas Field Station.

Both detection probability and concentration of N. topeka eDNA in water samples increased at a biomass density approximately two to three times that of the schools in which it naturally occurs (80 fish versus 25 to 30). An initial spike in concentration associated with initial stocking of fish was observed and concentrations increased with increasing stocking density (20, 40, and 80 individuals per tank). However, water column concentrations of fish eDNA decreased below detection limits within 7 days, regardless of stocking density. However after 14 days, the number of detections in the high density tank again climbed to levels comparable to the initial spike and remained at similar levels 26 days after stocking. *N. topeka* eDNA was detected in samples 27 days after fish removal, regardless of density, though with a lower detection probability than when fish were present. Despite consistent detection, water column concentrations of N. topeka eDNA did not exceed 20% of the initial spike over the course of the experiment (335 days).

Together, these experiments validate the potential use of eDNA for detection of both large-bodied and small-bodied fish in aquatic environments. Water column and surficial sediments both appear to be viable sources for recovery of fish eDNA, with greater concentrations and longer persistence in sediments, and increased biomass is generally associated with increased eDNA concentrations. Resuspension of sediments, especially those containing clays, and ecological changes associated with bioturbation and macrophyte removal appear to be important to both transport and fate of eDNA and may cloud biomass effects in both the water column and surficial sediments of aquatic systems. Future studies should focus on directly measuring functional drivers of degradation such as enzyme concentrations, ultraviolet light intensity, and relative concentrations of attached versus free eDNA. In addition, advanced modeling of the ecological conditions associated with water column and sediment eDNA could provide insight into the primary mechanisms that affect net eDNA concentrations and their change through time.

CHAPTER 1: A Framework for Understanding the Fate, Transport, and Applications of DNA Recovered from the Environment (eDNA)

INTRODUCTION AND MOTIVATION

Molecular methods have developed rapidly in the past decade, driven in large part by the need to understand the biology and dynamics of pathogen dispersal (Simpson et al. 2002, Rajal et al. 2007, Girones et al. 2010), mobile genetic elements (Gogarten and Townsend 2005, Venkata Mohan et al. 2009), and antibiotic resistance (Zhang et al. 2009, Kumarasamy et al. 2010). These methods all rely on the recovery of genetic material from the environment. Most applications for monitoring of this environmental DNA (eDNA) in environmental engineering and science have been limited to detection of microorganisms and their genes, but there are many higher-order species that have public health, ecological, and economic significance, including invasive species (e.g., zebra mussels, Asian carp) and declining species (e.g., snowy owl, mead's milkweed) that are critical to maintaining biodiversity. The potential applications of eDNA methods in ecology have been recognized for studying diet and disease, food web interactions, and changes in species distributions, niche stability, and biodiversity (Waits and Paetkau 2005, Taberlet et al. 2012, Yoccoz 2012). eDNA methods have also been adopted to monitor vertebrates in aquatic systems (Campbell et al. 2008, Ficetola et al. 2008, Goldberg et al. 2011, Jerde et al. 2011, Thomsen et al. 2012), including monitoring of biological invasions in aquatic environments (Darling and Mahon 2011, Takahara et al. 2013), and monitoring of endangered freshwater biodiversity (Thomsen et al. 2012, Fukumoto et al. 2015, Laramie et al. 2015, Spear et al. 2015). Existing studies have shown that collection, extraction, and amplification of vertebrate DNA from aquatic samples are possible. However, few studies have produced quantitative data in an attempt to move beyond presence/absence monitoring, and fewer of those have addressed fate and transport of vertebrate DNA once it enters the environment.

While existing studies suggest that eDNA sampling is a viable monitoring and surveillance approach for higher-order species, the quantitative methods and experimental data necessary to reliably relate the concentration of environmental DNA to concentration of living organisms are still under development. Answers to basic questions are still unknown, such as how long does aquatic vertebrate DNA persist in the environment, and where does it go? As potential applications grow more complex, the need for a fundamental understanding of the factors that affect the fate and transport of eDNA grows (Darling and Blum 2007, Bott et al. 2010, Darling and Mahon 2011, Taberlet et al. 2012, Goldberg et al. 2015). Moreover, DNA primers have been developed only for very few aquatic vertebrate species. Additional genetic sequencing and primer development will be required for widespread application. The goals of this work, therefore, are to contribute to an understanding of the factors affecting fate and transport of vertebrate DNA in the aquatic environment. Such understanding will allow both improved interpretation of quantification results and more confident connections between the concentration of vertebrate eDNA and the condition or population size of its parent organism.

Fate and transport of vertebrate DNA in the environment will be investigated using two model organisms: (1) a large-bodied invasive fish (bigheaded carps, *Hypophthalmichthys nobilis* and *Hypophthalmichthys molitrix*) and (2) a small-bodied endangered fish (Topeka shiner, *Notropis topeka*). These organisms were chosen to represent a range of potential target sizes for which vertebrate eDNA monitoring could be appropriate and for the broader impacts associated with these particular species (i.e., potential impacts to commercial fisheries in large-scale ecosystems for the invasive *Hypophthalmichthys spp.* and conservation implications for the federally-listed endangered *N. topeka*).

The central hypotheses of this work are threefold: (1) fish eDNA partitions into multiple compartments in aquatic environments (namely, water column, sediment, biofilms, and surface microlayers); (2) there is differential fate among these compartments, with fish eDNA concentrations both being a function of differential accumulation, decay, and transport and being consistently higher in sediments than the bulk water column; and (3) the net concentration of eDNA in a given waterbody is

reflective of the condition or population size of the parent organisms once fate and transport have been considered.

These three hypotheses were examined in several stages. First, tools were built to measure macrofaunal eDNA. Molecular markers, collection methods, extraction methods, and amplification methods were developed to amplify DNA of the endangered Topeka shiner (*Notropis topeka*) and the invasive Bigheaded carps (*Hypophthalmichthys spp.*) from environmental samples. Genetic data and markers for the Bigheaded carps had been previously developed (e.g., Jerde et al. 2011, Turner et al. 2014), but development of similar resources for Topeka shiners was required. We extracted DNA from vouchered tissue specimens, sequenced that DNA, produced novel sequence alignments, designed potential markers, performed polymerase chain reaction (PCR) and quantitative PCR (qPCR) testing on those markers, and developed sample collection and extraction methods for *N. topeka* DNA derived both from tissues and environmental samples. Second, we investigated the fate and transport of eDNA using microcosm and mesocosm samples via transport partitioning and fate experiments. The transport partitioning experiment to investigate the differences in concentration between water column and sediment eDNA concentrations has been published in a special issue of *Biological Conservation* (Turner et al. 2015). Since data on the degradation of eDNA in sediments are sparse, we collected sediments from an array of aquatic mesocosms containing different densities of target fish (*Hypophthalmichthys* spp.), then measured sediment eDNA concentrations in controlled microcosms over time. To test the third hypothesis that eDNA concentrations are related to organism parameters, we designed a mesocosm experiment to quantify the relationship between metrics of density, biomass, and number of *N. topeka* and water column *N. topeka* eDNA concentration over the course of one year.

BACKGROUND

eDNA monitoring of aquatic vertebrates is an emerging field (Figure 1), and basic questions regarding the partitioning, fate, and utility of eDNA in aquatic environments remain unanswered. The essential concept

behind eDNA monitoring is that organisms shed eDNA into the environment (Figure 2), that the eDNA can be recovered and measured, and that the measurement can provide information about organism presence or density (Figure 3a).

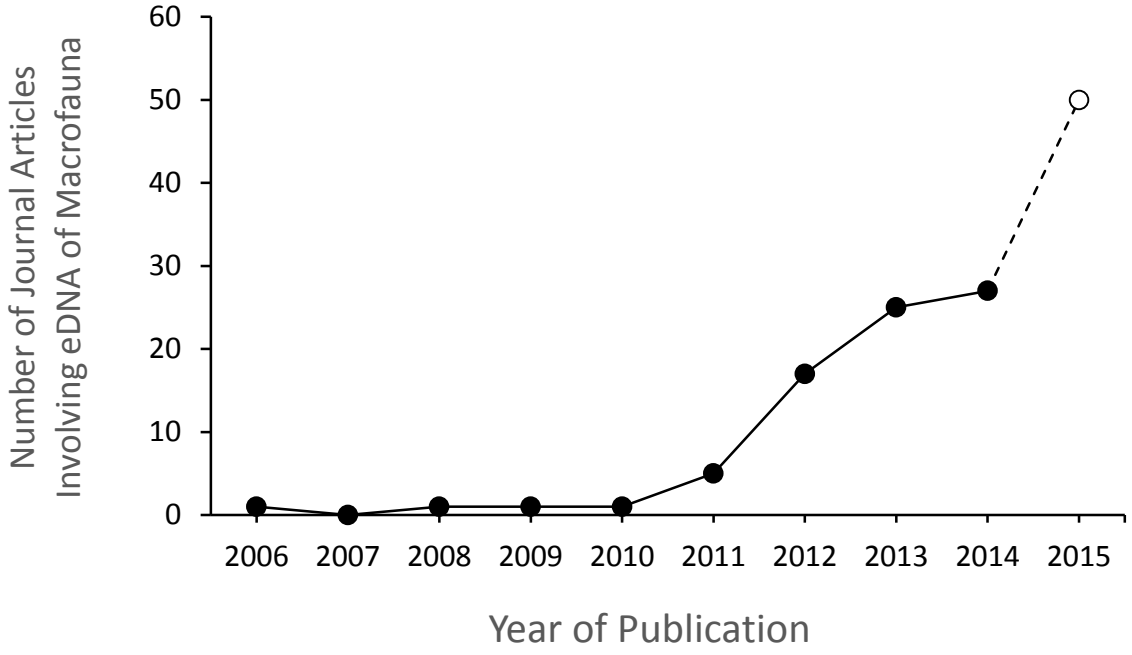

Figure 1. Peer-reviewed journal publications on environmental DNA of animal macrofauna. Counts are based on a Web of Science search for "environmental DNA," then screened for applications involving macrofauna. The count for 2015 is projected based on 25 publications through April of 2015.

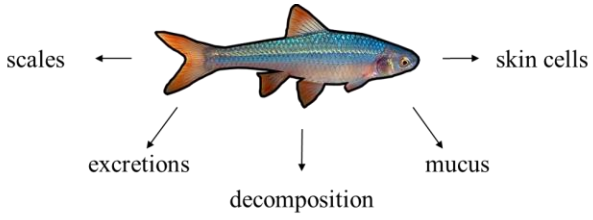

Figure 2. Sources of environmental DNA.

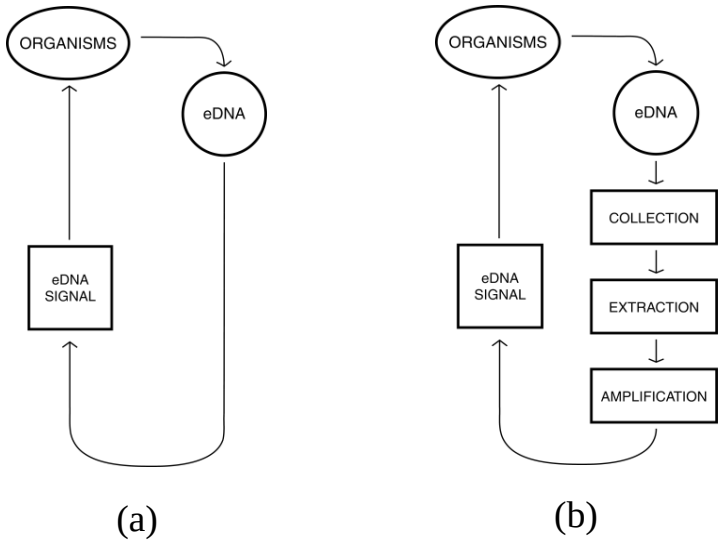

Figure 3. Environmental DNA monitoring (a) conceptual and (b) practical model.

In practice, eDNA must be collected from the environment, extracted from the collected sample, and then amplified to measure the signal (Figure 2b). Ogram et al. (1987) recognized that the DNA present in environmental samples is both intracellular (i.e., contained within cells) and extracellular (i.e., outside of cells). Unfortunately, a significant body of environmental engineering and microbiological literature refers to extracellular DNA as "eDNA." However, the term eDNA has more recently been used (and will be used throughout this work) to describe all DNA recovered from the environment, including both the extracellular and intracellular portions. In the case of macro-organisms like fish and other vertebrates, eDNA exists in a continuum of states in the environment: within organisms, within membrane bound structures outside of organisms (e.g., tissues, cells, and organelles), and outside of membranes (e.g., free floating or particle adsorbed extracellular DNA). Leff et al. (1992) recognized that both cells and DNA can spiral downstream in flowing freshwater systems, and in a review chapter on nucleic acids in soils, (Engemann et al. 2008) proposed the "extracellular gene pool hypothesis" that "continual release of DNA from organisms and tissue material in soil and its subsequent degradation with a time lapse will provide a

pool of extracellular DNA consisting of DNA from all kingdoms of life with DNA from microbial sources probably being the major fraction due to their high abundance." This hypothesis is the theoretical basis for eDNA monitoring.

In freshwater aquatic systems, eDNA from aquatic animals is dispersed in the water through methods such as the shedding of skin cells, scales, mucus, feces, urine, and the decomposition of organisms. Persistence of this eDNA in water depends on the rate of degradation of the DNA, which may vary from site to site due to differences in ultraviolet (UV) radiation, enzymatic hydrolysis, pH, and temperature (Matsui et al. 2001, Zhu 2006, Dejean et al. 2011, Barnes et al. 2014, Strickler et al. 2015). The use of eDNA to detect the presence of vertebrates in freshwater systems has been proven to be effective in wetland areas (Ficetola et al. 2008, Piaggio et al. 2014, McKee et al. 2015), small headwater streams (Goldberg et al. 2011, Thomsen et al. 2012), large rivers and canals (Jerde et al. 2011, Thomsen et al. 2012, Laramie et al. 2015), and lakes and ponds (Thomsen et al. 2012, Takahara et al. 2013). Overall, these recent studies have demonstrated the utility of molecular methods for monitoring the presence and absence of aquatic macrofauna. eDNA sampling is well suited for monitoring both endangered and invasive species, since the former are rare and often difficult to sample because of low numbers, sensitivity to handling, and avoidance behaviors, and management of the latter requires early detection before significant populations have become established. In such cases, an eDNA approach can potentially provide a faster, cheaper, and less invasive method for monitoring (Waits and Paetkau 2005).

ENVIRONMENTAL CONCENTRATIONS

Until recently, the majority of studies describing the location, concentration, fate, and transport of DNA in the environment have focused on the extracellular fraction of microbial DNA. Lorenz and Wackernagel (1994) published a significant review of the biology, presence, fate, transport, and potential for natural transformation of recombinant DNA in the environment, and several reviews have been published more recently on extracellular DNA in the environment in general (Nielsen et al. 2007, Tani

and Nasu 2010) and in soils in particular (Levy-Booth et al. 2007, Engemann et al. 2008, Pietramellara et al. 2009). Still, Thomsen and Willerslev (2015) have identified the continuing need for research on the "temporal and spatial distribution of eDNA in different habitats" and "more precise links between eDNA concentration and species abundance."

In general, extracellular DNA has been found in a range of aquatic environmental compartments including the water column, sediment, and biofilms in groundwater, surface freshwater, wetlands, estuaries, coastal waters, and open ocean (Figure 4). Sediment concentrations of extracellular DNA are greater than water column concentrations (roughly 10 times higher in marine systems and 10 to 500 times higher in freshwater systems). In the water column, coastal and estuarine systems have higher extracellular DNA concentrations than freshwater, followed by open ocean systems, but the opposite trend is observed in sediments. Deep ocean sediments have extracellular DNA concentrations from 4 to 10 times higher than freshwater sediments, and up to 10 times higher concentrations than terrestrial soils. Freshwater sediments contained up to 3 times less extracellular DNA than forest soils.

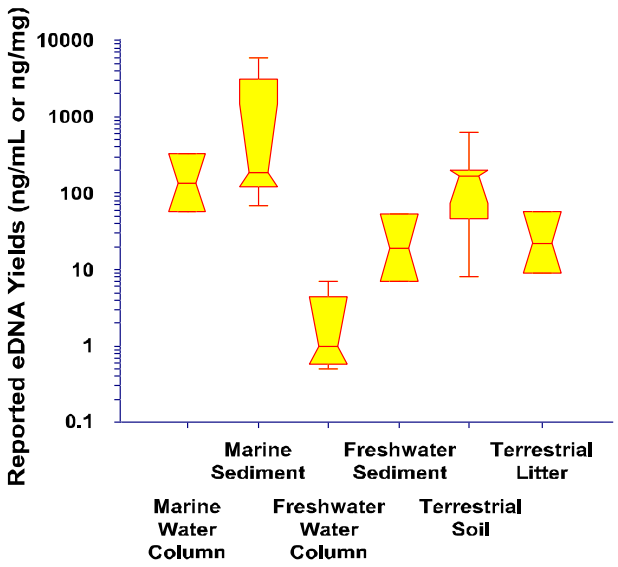

Figure 4. Reported concentrations of eDNA in various environmental compartments.
Data compiled from Pietramellara et al. (2009) and Corinaldesi et al. (2010). Middle bar indicates median value, upper and lower filled edges indicate the 3rd and 1st quartiles, respectively, and whiskers represent 1.5 x the interquartile range. Values beyond this range are considered outliers. Notches in the box plot represent the median +/- 1.57 x the interquartile range / the square root of the count. Where notches do not overlap, the medians are considered to be significantly different at the 95% level of significance.

Karl and Bailiff (1989) reported dissolved DNA concentrations in the water column roughly an order of magnitude higher in freshwater (1 to 90 ng/mL) and coastal/estuarine waters (0.7 to 80.6 ng/L) than in the open ocean (0.2 to 4.1 ng/mL), with all DNA passing through membrane filters with an average pore size of 0.05 um. Zhu (2006) has recently identified extracellular DNA in groundwater. DeFlaun et al. (1986) found dissolved DNA concentrations ranging from 0.4 to 1.75 ng/mL in ocean water, 10.8 ng/mL in coastal water, and 14.5 ng/mL in estuarine water, and observed concentrations in freshwater ranged from 1.7 to 7.8 ng/mL. Using a cetyltriammonium bromide extraction with chloroform separation modified from Pilliod et al. (2013), dissolved DNA concentrations from tanks containing *N. topeka* at the University of Kansas Field Station (KUFS) ranged from < 0.5 ng/mL to 870 ng/mL (author's unpublished data). Some of the higher range concentrations may result from either a higher density of

organisms than that of the DeFlaun et al. study, or a slight difference in the collection method (precipitation versus filtering), which might collect smaller DNA fragments more efficiently.

Improving on the methods of Ogram et al. (1987), Corinaldesi et al. (2005) developed a method to extract both intracellular and extracellular DNA separately from the same sample by first extracting the extracellular DNA, then extracting the intracellular DNA by lysing any remaining cells. Using this method, they found extracellular DNA concentrations 10 to 70 times higher than intracellular DNA concentrations in marine sediment samples. DNA is recoverable from marine sediments in concentrations ranging from 0.2 - 10.2 ng/mL in the open ocean to 2,000 - 58,000 ng/g in the top 10cm of deep, anoxic marine sediments, with recovered extracellular DNA fragments greater than 10 kb in length observed (Corinaldesi et al. 2011). Subsequent analysis of deep ocean, anoxic marine sediments have identified these sediments as major archives of eukaryotic gene sequences (Corinaldesi et al. 2011).

Extracellular DNA has been estimated as 10% to 60% of total DNA in a forest surface profile (Agnelli et al. 2004) and observed as 90% to 98% of total DNA in marine sediments (Corinaldesi et al. 2005). Paul et al. (1987) have estimated that 70% to 90% of extracellular DNA (i.e., DNA outside cell membranes) in the marine water column is due to bacterioplankton. Dell'Anno et al. (2002) found that concentrations of extracellular DNA generally declined with soil depth and that hydrolyzable DNA in sediment samples comprised a wide range of total DNA (less than 10% to greater than 70%), depending on the nature, location, and depth of the sediment sample. Dell'Anno et al. (2002) determined that >95% of recoverable extracellular DNA in marine sediments was bound to the sediment matrix, and more than 50% of the extracellular DNA present in the top 15 cm was recalcitrant to enzymatic degradation, suggesting potential for resuspension of transformable DNA and a non-negligible role for DNA in phosphorus dynamics.

DEGRADATION

Levy-Booth et al. (2007) proposed a cycle for extracellular DNA in soils with three potential fates: (i) binding to the soil matrix, (ii) breakdown by DNase sequence restriction, or (iii) natural

transformation into soil organisms. DNA enters this cycle by addition of DNA released by organisms (both living and nonliving), and exits the cycle by removal of DNA through uptake by organisms or reincorporation into other macromolecules. In aquatic ecosystems, eDNA from aquatic animals is dispersed through the water (Figure 5). Therefore, the DNA may float, sink, or adhere to surface films, depending on degradation and transport rates. DNA in aquatic systems may have analogous potential fates: (i) binding to suspended or precipitated particles, biofilms, or surface foams, (ii) breakdown by physical, photic, chemical, radioactive, or biological means, or (iii) natural transformation into aquatic organisms.

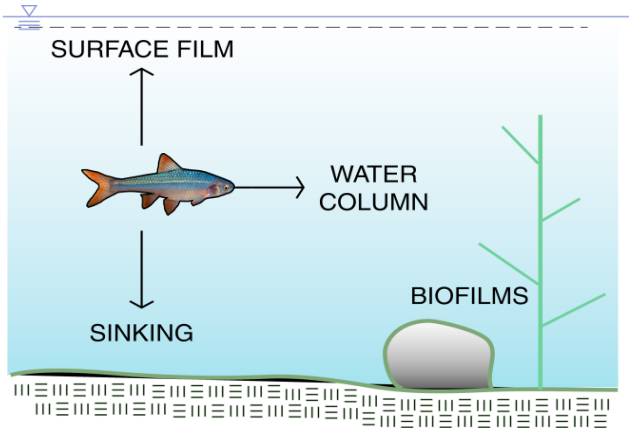

Figure 5. Proximal transport partitioning of environmental DNA from source organism.

DNA breaks down by hydrolysis, oxidation, and nonenzymatic methylation (Lindahl 1993) *in vivo*, and is subjected to physical (e.g. temperature, ultraviolet light), chemical factors (e.g., water, redox and pH changes) and biological factors once outside the cell (Shapiro 2008). Radioactivity can also cause double strand breaks of extracellular DNA in freshwater (Arruda-Neto et al. 2012). In terrestrial systems the expected primary mechanism of DNA breakdown is depurination, but hydrolytic restriction by nucleases has been shown to be the primary degradation mechanism of extracellular DNA in aquatic systems (Paul et al. 1987). For reference, commercially available deoxyribonuclease I (DNase I) can hydrolyze DNA at a rate of 6 x 10^6 ng /L per hour per mg of enzyme, and ambient concentrations of

DNases sufficient for hydrolysis have been observed in the environment (Paul et al. 1987). Observed DNase activity has been as high as 1240 ng DNA degraded per gram of soil per hour in deep ocean, anoxic sediments, where activity increases with water depth (Corinaldesi et al. 2011). It is this degradation and restriction of DNA sequences that renders reconstruction of the original genetic sequence increasingly difficult with the passage of time (Shapiro 2008).

Degradation rates of extracellular DNA have been published for many aquatic environments (Figure 6) (see also Nielsen et al. 2007), but the fate of vertebrate DNA in aquatic systems is less understood. Rapid hydrolysis of extracellular DNA by cell-associated and extracellular nucleases has been observed in both marine (Paul et al. 1987) and freshwater (Matsui et al. 2001) environments. Since extracellular DNA is a substrate for the DNase enzyme, Paul et al. (1987) used a Michaelis-Menton kinetics approach to determine parameters of enzyme-based degradation of extracellular DNA. Based on observed data and the assumptions that (i) dissolved bacterioplankton were at a steady-state; and (ii) dissolved bacterioplankton were the only source of extracellular DNA, Paul et al. (1989) calculated that DNA had a turnover time ranging from 22.7 to 146 days in sheltered harbors versus 32.3 days in open ocean water, and less than 6.5 hours in estuarine waters. Particulate material was believed to be the likely site of most active microbial macromolecular degradation.

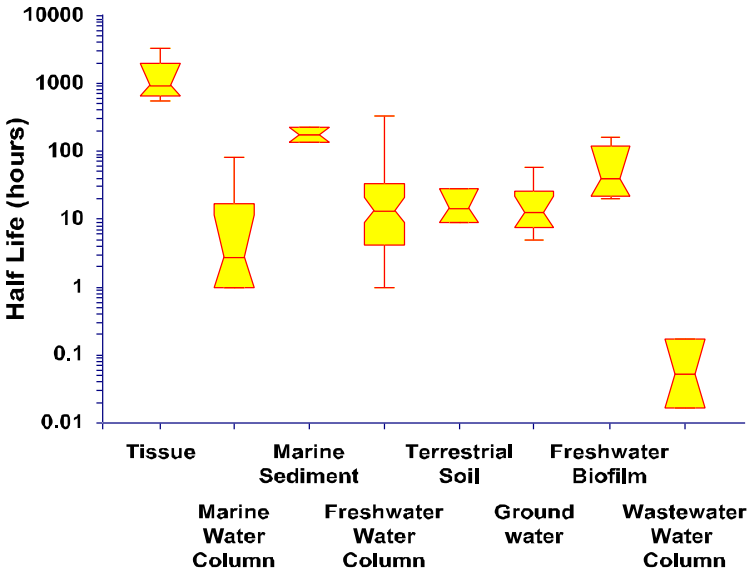

Figure 6. Reported persistence of eDNA in different environmental compartments
Data compiled from Lorenz & Wackernagel (1994), Engemann et al. (2008), Zhang et al. (2009), Engemann et al. (2006), Dejean et al. (2011), Schnell et al. (2012), Zhu (2006), Alvarez et al. (1996), Matsui et al. (2001), Fu et al. (2012), and Allentoft et al. (2012). Middle bar indicates median value, upper and lower filled edges indicate the 3rd and 1st quartiles, respectively, and whiskers represent 1.5 x the interquartile range. Values beyond this range are considered outliers. Notches in the box plot represent the median +/- 1.57 x the interquartile range / the square root of the count. Where notches do not overlap, the medians are considered to be significantly different at the 95% level of significance.

Dell'Anno and Corinaldesi (2004) found that degradation rates for extracellular DNA in sediments were 7 to 100 times higher than those for extracellular DNA in the water column. However, significantly higher concentrations of extracellular DNA in sediments resulted in turnover times 64 to 223 times lower than those for the water column (29 to 93 days versus 0.41 days). Extracellular DNA concentrations in sediments were 4.3 times higher on average than concentrations of DNA associated with total bacterial cells (assuming 3.3 fg DNA/cell), indicating that the majority of the extracellular DNA pool is not accounted for by living biomass. Further, assuming an average phosphorus content of DNA at 10% and a bacterial C:N:P ratio of 40:10:1, they calculated that extracellular DNA could potentially supply 41% of the daily bacterial P requirements for the average condition of the 3 sample sites.

Corinaldesi et al. (2008) found that >90% of the variance in extracellular DNA variance in marine sediments was explained by water depth (34%), biopolymeric organic carbon content (25%), temperature (14%), salinity (12%), and redox potential (6.5%), where biopolymeric carbon is the sum of protein, carbohydrate, and lipids in carbon equivalents (a proxy for organic matter). They also found that depurination processes of DNA in marine sediments cannot be predicted by temperature alone. DNase-mediated degradation was not coupled with depurination processes, and both chemically- and biologically-driven decay rates were important.

Dell'Anno et al. (2002) observed seasonal changes in the ratios of the deoxynucleoside products of enzymatic hydrolysis of extracellular DNA. Molar ratios of 2'-deoxycytidine (dC), 2'-deoxyguanosine (dG), 2'-deoxythymidine (dT), and 2'-deoxyadenosine (dA) may reveal the relative timeframe of introduction of external sources of extracellular DNA into a system. Since molar ratios of dC:dG and dA:dT close to 1 were associated with large inputs of primary organic material from the photic layer, shifts in this molar ratio have been suggested to be indicative of selective degradation and/or utilization of extracellular DNA (Dell'Anno et al. 2002). Similarly, higher copy numbers of 18S rDNA gene sequences may reflect temporal changes of DNA inputs from the water column and progressive accumulation in the sediments (Corinaldesi et al. 2011).

Few eDNA degradation rates have been published for vertebrate eDNA aquatic systems. Dejean et al. (2011) found that Siberian sturgeon (*Acipenser baerii*) and bullfrog (*Lithobates catesbeianus*) eDNA degraded in tanks and ponds within 3 and 4 weeks, respectively, with lower temperatures slowing microbial activity and the rate of eDNA degradation. Thomsen et al. (2012) used qPCR and next generation sequencing to monitor a suite of macroinvertebrate, amphibian, fish, and mammal species in freshwater mesocosms, streams, and ponds. They developed a model for degradation using data from mesocosm studies of two amphibians (*Pelobates fuscus* and *Triturus cistatus*), and observed rapid degradation of eDNA with non-detection at 1-2 weeks after removal of the source organisms. Barnes et al. (2014) estimated the exponential degradation rate r (where $[eDNA] = 32.164e^{-r t}$) of Common Carp eDNA in experimental aquatic microcosms to be 0.105 +/- 0.014 1/hr (mean +/- 1 SE), and found that

degradation rate decreased with increasing pH, chlorophyll a concentration, biochemical oxygen demand, and total eDNA concentration. The decreased rate was hypothesized to result from decreased degradation by UV or preferential degradation of other substrates (goldfish waste) by microbes. Pilliod et al. (2014) studied the production, degradation, and detectability of environmental DNA from salamanders under varying environmental conditions. They found that eDNA from salamanders in water degraded exponentially to less than 1% of the original concentration in both full sun and shaded treatments after 3 days; and by 11 days, the proportion of detections was zero in full sun treatments and 0.2 in shade treatments. Less than 2% of the original concentration in refrigerated control treatments within 18 days, but eDNA was still detected in 100% of refrigerated control samples.

Based on experimental treatments of temperature, UV-B, and pH, Strickler et al. (2015) found that bullfrog (*Lithobates catesbeianus*) eDNA degrades more rapidly in aquatic systems that are amenable to microbial growth (warmer, neutral pH), brighter, or more acidic, and persists longer in colder, darker, alkaline conditions. Exponential degradation rates ranged from 0.34 1/hr in high temperature, high intensity UV-B, low pH (35degC, 50 kJ/m^2/day, 4) treatments to 0.05 1/hr in low temperature, low intensity UV-B conditions (5degC, 2 kJ/m^2/day), at both low and high pH (4 and 10, respectively). Based on these degradation rates, detectability of bullfrog eDNA ranged from less than 1 day to at least 58 days.

Jane et al. (2015) introduced caged brook trout (*Salvelinus fontinalis*) into fishless, high-gradient, headwater streams and measured eDNA concentration downstream at different distances and flows. eDNA was detectable within 24 hours of introduction and at up to 239.5m downstream from the cages in all samples, but 24 hours after removal of the source fish, most eDNA was not detectable in either stream. Observed stream-dependent variation in eDNA dynamics were attributed to presumed differences in physical properties such as shear stress, bed roughness, and transient storage zone distribution (Jane et al. 2015).

As exhibited by these studies, vertebrate eDNA does persist in the environment and can degrade within a relatively short timeframe. Therefore, eDNA could be an effective indicator of recent species occurrence. Moreover, variable environmental conditions could explain differences in reported eDNA

degradation rates observed in multiple studies (Barnes et al. 2014). In order to relate the concentration of DNA to the concentration of living species, then, it is necessary to understand both the rate of DNA degradation as a function of the chemical and physical properties of a given environmental sample and the source and relative contribution of different states of eDNA (e.g., intra-cellular, free-floating, or surface-adhered) to the observed signal (Thomsen et al. 2012, Turner et al. 2014, Turner et al. 2015).

PROTECTION FROM DEGRADATION

Attachment to soils, sediments, and suspended material has been shown to decrease the degradation rate of extracellular DNA. Mineral and organic adsorption of eDNA limits availability for DNase attack (Coolen and Overmann 2007), and DNase activity is also reduced by DNase adsorption to sediment particles (Demaneche et al. 2001). Binding to the soil matrix reduces eDNA degradation by protecting DNA from nuclease-mediated hydrolyzation (Crecchio and Stotzky 1998, Levy-Booth et al. 2007), and the soil matrix can also bind DNases and other nucleases, effectively reducing the amount of nucleases available to mediate hydrolyzation (Blum et al. 1997, Levy-Booth et al. 2007). Binding of DNA to soil is controlled in part by the intrinsic surface area of the soil itself. Clay has 3 orders of magnitude more surface area per weight than sand (Romanowski et al. 1992, Lorenz and Wackernagel 1994, Blum et al. 1997, Levy-Booth et al. 2007), and the binding capacity of a particular soil is therefore determined by clay content (Levy-Booth et al. 2007). DNA binding to soil is also controlled in part by pH. The isoelectric point of DNA occurs at pH 5, with lower pH resulting in protonation of DNA (Levy-Booth et al. 2007, Theng 2012). The net positive charge of DNA at pH below 5 allows for direct adsorption to soil constituents. However, since both DNA and soil typically have net negative charges above pH 5, cations are required to facilitate DNA adhesion to the soil matrix (Levy-Booth et al. 2007, Minamoto et al. 2012). Lorenz and Wackernagel (1987) observed increased adsorption of DNA to sand at low pH (<6) and at high pH (>8) in the presence of divalent magnesium. Crecchio et al. (2005) observed adsorption of extracellular DNA to iron and aluminum organomineral complexes characteristic of humic acids, and subsequent protection of the adsorbed DNA from hydrolysis by DNase. DNA was

also found not to desorb in the presence of EDTA, detergents, and NaPO4 and NaCl solutions, and higher divalent cation concentrations have been associated with higher rates of adhesion (Romanowski et al. 1992, Levy-Booth et al. 2007).

Blum et al. (1997) reported a large portion of extracellular DNA adsorbed to three different soils was of relatively high molecular weight (from several hundred to several thousand base pairs in length). Extracellular DNA was shown to leach from the soil back into the interstitial pore water for 24 hours, where acid hydrolysis was performed by DNases in solution. Growing prokaryotes were suggested to be the main producers of these DNases. Crecchio et al. (2005) observed that though DNase restriction did not occur to adsorbed DNA, transformation did, suggesting that bound DNA may still provide genetic information even when still adsorbed.

SEDIMENT RESUSPENSION, BIOFILMS, AND SURFACE FILMS

Leff et al. (1992) predicted downstream transport of cells and genetic information (i.e., DNA both within and outside of cells), and Jamieson et al. (2005) noted resuspension of *E. coli* in a natural stream with sufficient stream flow to cause shearing of cohesive sediments. Dell'Anno et al. (2002) have observed a rapid decrease in eDNA concentration with depth in undisturbed marine sediments, and Agnelli et al. (2004) have identified downward movement of eDNA in forest soil profiles. However, Corinaldesi et al. (2008) have found that in sediments with continuous mixing, DNA degradation was constant with depth and therefore age of sediment may not be predictive of DNA degradation. Moreover, Dell'Anno et al. (2002) observed that >95% of eDNA in sediments may be attached to the soil matrix and >50% of sediment eDNA may be protected from DNase hydrolysis by that attachment. These observations imply not only that resuspension of soil particles would likely result in resuspension of eDNA, but also that changes in state of resuspended sediment particles could lead to subsequent release of undegraded eDNA.

The fate of high molecular weight DNA in the environment can also be affected by environmental biofilms. Research has shown that tetracycline resistance gene determinants degrade about

1.4 times faster in sunlight than in the dark (Engemann et al. 2006), that tetracycline resistance genes differentially migrate into biofilms (Engemann et al. 2008), and that disappearance of the genes was observed to be twice as slow in biofilms as in the water column (Engemann et al. 2008). Zhang et al. (2009) found that >85% of the total tetracycline resistance genes detected in freshwater mesocosm samples were associated with biofilms within 4 days of a single pulse addition of sediment containing the genes. The half-life of these genes was up to 5 times longer in biofilms when compared to the water column. Additionally, Bockelmann et al. (2006) reported that the microfilament structure produced by an aquatic bacterium was composed predominantly of extracellular DNA, with the majority of fragments in the 5000bp range, and Harmsen et al. (2010) found that DNA fragments greater than 200bp in length can combine with peptidoglycan to form structural biofilm polymers required for adhesion to surfaces and other cells by some organisms.

Surface films and foams may also have relationships with eDNA. A surface microlayer, typically one millimeter in thickness, exists at the boundary between surface waters and the atmosphere (Cunliffe et al. 2011, Theng 2012, Cunliffe et al. 2013). This microlayer is an environmental compartment that is distinct from the bulk water column and characterized by different physical, chemical, and biological conditions (e.g. surface tension and adhesion, concentration of hydrophobic compounds compared to bulk water column, and biofilms resistant to ultraviolet light, respectively) (Cunliffe et al. 2013, Spear et al. 2015, Strickler et al. 2015). Sigsgaard et al. (2015) have also noted associations of fish eggs and larvae with the sea surface microlayer. Though minimal in thickness, the volume of the aquatic surface microlayer can be significant in waterbodies with large areal extent or relatively high surface to volume ratios, and is conservatively estimated at 3.3×10^8 m^3 worldwide (Theng 2012). Given their higher surface to volume ratio, shallow waterbodies likely have closer coupling between the surface microlayer, the water column, and the benthic sediments (Theng 2012). Moreover, recent evidence suggests that even though the sea surface microlayer can be easily disturbed by physical disruption, reestablishment of the microlayer occurs on the order of minutes.

Transparent exopolymer particles (TEP) are dissolved, sticky, gel-like substances formed primarily from polysaccharides produced in the mucus of phytoplankton, corals, and other organisms (Laramie et al. 2015). These substances are ubiquitous in aquatic systems and provide substrate for adhesion of suspended particles, bacteria, and trace solutes (Laramie et al. 2015). Further, TEP and their adhered and associated compounds have been shown to be both buoyant (McKee et al. 2015) and enriched in sea surface microlayers (Takahara et al. 2015). In a recent review, Cunliffe et al. (2011) have suggested that similar TEP mediated transport processes are likely in freshwaters.

DeFlaun et al. (1986) have reported dissolved DNA concentrations 2 to 12 times higher in coral mucus than in the overlying water. Suzuki and Maruyama (2000) found that fish mucus in alkaline (pH 10) aqueous solutions was associated with 99% removal of suspended kaolin clay particles in seawater. Clay particles such as these have been associated with attached environmental DNA in the water column (Paul et al. 1987, Theng 2012). Nguyen and Chen (2007) found that divalent cations, especially Ca^{2+}, promote adsorption of DNA to silica particles coated with a natural organic film. These results suggest potential for formation of surface films that both contain and adhere free-DNA and DNA-carrying particles.

Taken all together, these phenomena have implications for eDNA monitoring of vertebrates. Accurate quantification of target organisms from eDNA will need to account for the fate and transport of eDNA in the environment, including source production, degradation, transport partitioning (i.e., to particle-attached, free-floating, and film-associated phases), and potential resuspension or release from attached phases.

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CHAPTER 2: Development of Novel Genetic Markers for the Detection and Potential Quantification of Topeka Shiners (*Notropis topeka)*

INTRODUCTION

The Topeka shiner is a small minnow currently listed as endangered by the U.S. Fish and Wildlife Service (Federal Register 1998) (Figure 1). Historically, this small minnow was widespread and abundant throughout many headwater streams of the central prairie regions of the United States, but the known range of this species has been reduced by nearly 90% in the past 50 years (Federal Register 2005). Remaining wild populations of Topeka shiners inhabit small tributary streams – primarily in the Kansas and Cottonwood river basins in Kansas and in a few restricted watersheds in Missouri, Iowa, Nebraska, South Dakota, and Minnesota (http://www.fws.gov/mountain-prairie/species/fish/shiner/).

(a)

(b)

Figure 1. The Topeka shiner (Notropis topeka) (a) and its closest genetic relative, the sand shiner (Notropis stramineus).
Both are freshwater cyprinids with adults generally ranging 55 to 100 mm in total length. N. topeka is a federally listed endangered species with habitat formerly ranging across Kansas, Nebraska, Missouri, Iowa, South Dakota and Minnesota. *N. stramineus* is common and inhabits the same range. N. topeka photo courtesy of Garold Sneegas and the Kansas Biological Survey. *N. stramineus* illustration by Ellen Edmonson and Hugh Chrisp courtesty of the New York State Department of Environmental Conservation.

The approved Kansas Recovery Plan for the Topeka shiner (Mammoliti 2004) requires resource managers to "continue genetic studies to define population boundaries and genetic limitations that may impact the species," and to "develop a plan to implement long-term monitoring of populations and

habitats." The recovery plan additionally calls for wildlife managers to "monitor annually Topeka shiner populations and instream habitats within all occupied habitats," to "initiate reintroduction efforts in suitable, non-occupied habitats," and to "monitor all reintroduced populations to determine success/failure." Since endangered species often occur in very low abundances, using eDNA to detect the presence or absence of these species can be a very useful and cost-effective survey tool (Goldberg et al. 2015). eDNA survey methods (either presence/absence or quantitative) are also non-invasive and do not require the capture or return of organisms to assess their occurrence within targeted areas (e.g. stream segment, pond), affording a potentially safer approach (i.e. no capture or release) and reducing or eliminating habitat disturbances (Thomsen and Willerslev 2015). Additionally, eDNA sampling has the potential to detect organisms at very low densities even in complex habitats where other sampling techniques may be less efficient. (Thomsen and Willerslev 2015).

In line with federal directives, the Kansas Biological Survey (KBS) has maintained two genetically distinct subpopulations of Topeka shiners at the University of Kansas Field Station (KUFS) since 2002 (Campbell 2010), where the fish are maintained for conservation, potential breeding, and long-term study to benefit the species. Using mitochondrial DNA from these and other fish, Michels (2000) indicated that there are three distinct genetic groups of Topeka shiners – one in the Arkansas River drainage, the second in the Kansas and lower Missouri River drainages, and the third in the upper Missouri and Des Moines River drainages. A few recent studies have focused on Topeka shiner DNA (Schmidt and Gold 1995, Michels 2000, Anderson and Sarver 2008), but no qPCR markers have been developed for environmental monitoring, and little sequence information is available for the two Topeka shiner populations (Deep Creek and Willow Creek) currently in culture at KUFS. Though several microsatellite primers have been investigated for population variation (Anderson and Sarver 2008), their ability to cross amplify close taxanomic relatives (i.e. sand shiner, *Notropis stramineus*) has not been tested. Moreover, to date, no known species-specific primers for *N. topeka* have been published.

Althought the entire mitogenome of *N. stramineus* has been sequenced (Figure 2), only 5 *N. topeka* DNA sequences for the mitochondrially encoded cytochrome oxidase I gene (COI), 3 for the

mitochondrially encoded cytochrome oxidase II subunit B gene (CytB), and zero for the mitochondrially encoded NADH dehydrogenase 2 gene (ND2) have been recorded in the National Institute of Health's GenBank (http://www.ncbi.nlm.nih.gov/genbank/), and no known additional sequences of these genes for *N. topeka* have been published. These genes are all involved in cellular respiration and are generally present across a wide variety of organisms, allowing for potential discrimination between *N. topeka* and other organisms. The COI gene is commonly used for "barcoding" and other systematics and taxonomic studies, because it is relatively conservative at the genus level, but relatively variable at the species level. The CytB and ND2 genes exhibit similar properties. Recent vertebrate studies have used CytB for detection of bullfrogs {Ficetola, 2008 #692}, Rocky Mountain tailed frogs and Idaho giant salamanders {Goldberg, 2011 #713}, freshwater fish , and marine fish (Thomsen et al. 2012). Since COI is a commonly used target gene for taxonomic investigation (Ward et al. 2009, Shokralla et al. 2011), there is a fairly robust collection of previously sequenced COI from other fish species, including those commonly found in similar environments. However, sequences for CytB and ND2 for other species were not as readily available. Therefore, further genetic sequencing of *N. topeka* and development of molecular methods for identifying and potentially describing populations of *N. topeka* is not only necessary, but will provide the basis for the eDNA analyses in this work and will also directly contribute to conservation efforts at KUFS and the mandate for long-term monitoring and preservation of the species.

In order to design primers for any species, DNA sequences from the species of interest must be known and compared to sequences from the same region in non-target species. The KU Natural History Museum has an extensive collection of preserved fish specimens available for use, thus avoiding any need for collection of new specimens of endangered species as an initial tissue source.

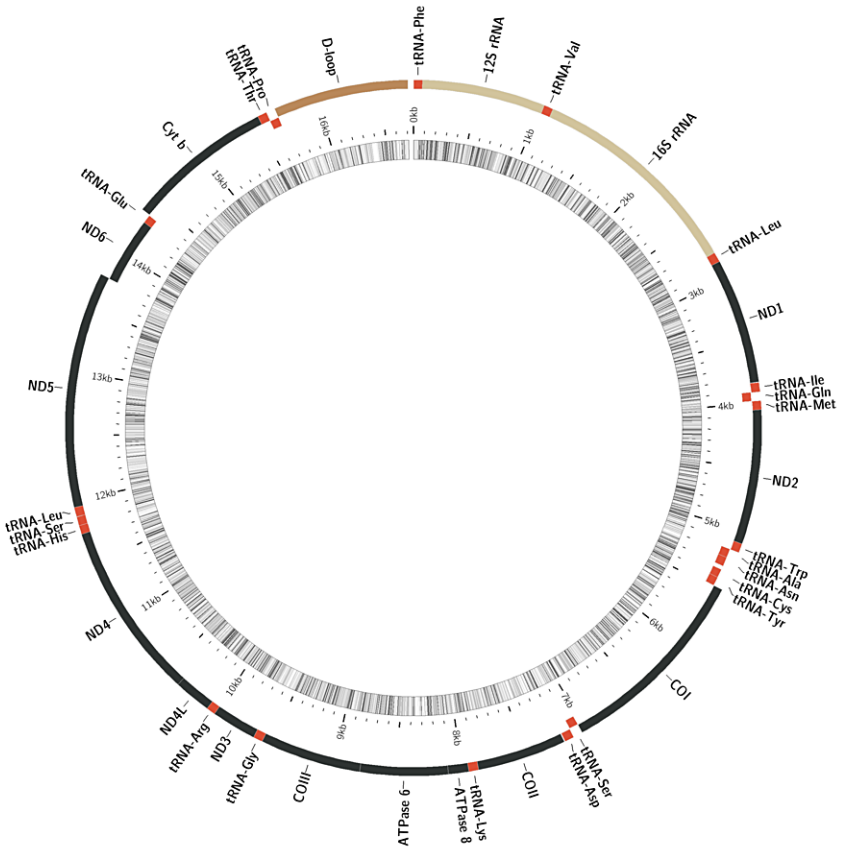


Figure 2. Mitogenome of the sand shiner (*Notropis stramineus*).
N. stramineus is the closest relative of the Topeka shiner (*Notropis topeka*). Regions sequenced in this study include the mitochondrially encoded genes cytochrome oxidase I gene (COI), cytochrome oxidase II subunit B gene (CytB), NADH dehydrogenase 2 gene (ND2) have, and the non-coding control region (D-loop). Inner-most circle represents percent GC content of every 5 base pairs, with darker lines having higher GC content. Sequence originally published by Broughton and Reneau (2006), then visualized using MitoFish (Iwasaki et al. 2013).

METHODS

Initial Tissue Extractions

Tissues used from the museum collection are summarized in (Table 1) with the number of specimens available, and the number of specimens extracted for this work. In order to design robust

primers, it is essential to align sequences of many individuals within the same species, so that primers can target conserved regions. However, it is also necessary that sequences in these conserved regions be sufficiently different than the conserved regions of closely related species. By extracting DNA from a large number of individuals spanning the full geographic range of Topeka shiner populations (i.e., multiple individuals from all three distinct genetic populations), we were able to design robust primers that consistently identify Topeka shiner mitochondrial DNA sequences.

Table 1. KU Natural History Museum fish tissues involved in this study.
All of the individuals listed are vouchered specimens from the museum collection. Therefore, no additional tissue samples were required, and no additional fish were collected for tissue sequencing.

Common Name	Scientific Name	Number Available from Collection	Number Extracted and Sequenced
Topeka shiner	*Notropis topeka*	186	186
Sand shiner	*Notropis stramineus*	94	10
Mimic shiner	*Notropis volucellus*	1	1
Rosyface x Common shiner hybrid	*Notropis rubellus x Luxilus cornutus*	1	1
Orangespotted sunfish	*Lepomis humilis*	7	1
Green sunfish	*Lepomis cyanellus*	2	1
Red shiner	*Cyprinella lutrensis*	40	1
Redfin shiner	*Lythrurus umbratilis*	4	1
Central stoneroller	*Campostoma anomalum*	183	1
Common creek chub	*Semotilus atromaculatus*	175	1
Largemouth bass	*Micropterus salmoides*	23	1
Bluntnose minnow	*Pimephales notatus*	5	1

Laboratory Methods

Sample processing was compartmentalized to limit accidental contamination. High-copy extractions (e.g. tissue) and low-copy extractions (e.g., environmental) were performed in physically separate laboratories, and separate pre- and post-PCR processing of samples was strictly observed. Lo-bind plastics and filter tip pipettes were used to limit template loss and cross contamination, and sample storage conditions followed recently developed suggestions from the literature (e.g., long term storage at -80 °C, minimization of number of freeze/thaw cycles) (Takahara et al. 2015).

180 of the 186 available *N. topeka* tissue samples were extracted, and sequence databases for 80 of these were developed for three genes (COI, CytB, and ND2). DNA extracts that were not sequenced have been reserved for validation testing. From the KU Natural History Museum's collection, 26 individuals of 12 co-occurring species were extracted, quantified by Nanodrop spectrophotometer, and cycle sequenced (Sanger et al. 1977) for COI, CytB, ND2, and the mitochondrial control region (D-loop) (Table 2). Additional sequences used for *in silico* testing of our markers against COI, CytB, and ND2 were retrieved from GenBank (Table 2). All novel sequence data from this dissertation will be uploaded to GenBank for public use.

Table 2. Species used in design of primers for *Notropis topeka.*
The Topeka shiner is the target of the assay, the sand shiner is the closest related non-target species, and the remaining are all potentially co-occurring species.

Common Name	Scientific Name	Gene of Interest			Sequence Source	
		COI	CytB	ND2	This Study	GenBank
Topeka shiner	*Notropis topeka*	x	x	x	x	x
Sand shiner	*Notropis stramineus*	x	x	x	x	x
Black bullhead	*Ameiurus melas*	x				x
Central stoneroller	*Campostoma anomalum*	x	x	x	x	
Red shiner	*Cyprinella lutrensis*	x	x	x	x	
Blacktail shiner	*Cyprinella venusta*	x	x			x
Green sunfish	*Lepomis cyanellus*	x	x	x	x	
Orangespotted sunfish	*Lepomis humilis*	x	x	x	x	
Common shiner	*Luxilus cornutus*	x			x	
Redfin shiner	*Lythrurus umbratilis*	x	x	x	x	
Largemouth bass	*Micropterus salmoides*	x	x	x	x	
Golden shiner	*Notemigonus crysoleucas*	x	x			x
Rosy face shiner	*Notropis rubellus*	x		x	x	
Mimic shiner	*Notropis volucellus*	x	x	x	x	
Suckermouth minnow	*Phenacobius mirabilis*	x				x
Bullhead minnow	*Pimephales notatus*				x	
Bluntnose minnow	*Pimephales vigilax*	x	x	x	x	
Common creek chub	*Semotilis atromaculatus*	x	x		x	
Channel catfish	*Ictalurus punctatus*		x			x
Rainbow shiner	*Notropis chrosomus*		x			x
Swallowtail shiner	*Notropis procne*		x			x
Emerald shiner	*Notropis atherinoides*			x		x

COI denotes the mitochondrially encoded cytochrome oxidase I gene
CytB denotes the mitochondrially encoded cytochrome oxidase II subunit B gene
ND2 denotes the mitochondrially encoded NADH dehydrogenase 2 gene

Vouchered museum specimens, typically tissues stored in ethanol and held at -80 ºC, were used for initial sequencing. Tissue extractions were carried out using a standard protienase K protocol. Small masses of tissue (approximately 10 mg) were dissolved in 600uL lysis buffer (100mM NaCl, 100mM Tris-HCl pH 8.0, 25mM EDTA pH 8.0, 0.5% sodium deodecyl sulfate) with 3-5uL of 20mg/mL proteinase K at 55 ºC, and were heated and shaken periodically until tissues were fully dissolved (typically overnight). The dissolved tissues were allowed to cool to room temperature, then proteins

were precipitated by addition of 4M guanadine thiocyanate and 0.1M Tris-HCl, followed by vortexing, centrifugation, and subsequent addition of chilled 100% isopropanol. Extracts were then spun down, the pellets were rinsed with 70% ethanol and spun again, and the ethanol was decanted. After evaporation of the ethanol, samples were resuspended in 10mM Tris-HCl (pH 8.0) and stored at -20 ºC for future use.

Extracted samples were PCR-amplified for 4 gene regions (COI, CytB, ND2, and the mitochondrial control or D-Loop region) using previously published generic markers for fish mitochondrial DNA (Pramuk et al. 2007, Cheng et al. 2012) (Table 3). These PCR products were quantified by nanodrop spectrophotometry, then cleaned using a commercial kit, USB ExoSAP-IT (Affymetrix, Inc. USA), to remove unused primers and nucleotides from the PCR products that could interfere with sample processing downstream. Samples were subsequently bidirectionally sequenced using an Applied Biosystems 3730 DNA Analyzer and contiguous sequences were formed from chromatogram traces using FinchTV software (Geospiza, Inc. USA, http://www.geospiza.com/finchTV).

Table 3. Sequencing (s) and amplification (a) primers used in this study.
Primer characteristics analyzed using Integrated DNA Technologies (IDT) software OligoAnalyzer 3.1. Sources of primer sequences are: (1) this study (RCE), (2) this study (MJG), (3) Pramuk et al. (2007), (4) Cheng et al. (2012). Targeted genes were cytochrome oxidase b (CytB), cytochrome oxidase I (COI), NADH dehydrogenase 2 (ND2), and the non-coding mitochondrial DNA control region (D-loop).

Source	Primer Name	Targeted Region	Sequence (5' to 3')	Length (base pairs)	Percent GC Content	Molecular Weight (g/mol)	Melting Temp (ºC)
1a	NtopCytb 498F	CytB	AGGCTTTTCGGTGGATAACGCGACG	25	56.0	7738.0	70.3
1a	NtopCytb 648F		CAAAATTTCCTTCCACCCCTACTTCTCTTAC	31	41.9	9792.4	58.8
1a	NtopCytb 558F		GTTCGTCATTGCCGGTGCAACG	22	59.1	6742.4	68.5
1a	NtopCytb 579R		CGTTGCACCGGCAATGACGAAC	22	59.1	6729.4	68.5
1a	NtopCytB 678R		GTAAGAGAAGTAGGGGTGGAAGGAAATTTTG	31	41.9	9792.4	58.8
1a	NtopCytb 742F		CACTACTCGGTGATCCAGATAAC	23	47.8	6992.6	62.0
1a	NtopCytb 765R		GTTATCTGGATCACCGAGTAGTG	23	47.8	7094.6	62.0
2a	Cytb F Ntop		TCCGTTCGTCATTGCTGGTGCAACG	25	56.0	7640.0	70.9
2a	Cytb R Ntop		ATCACCGAGTAGGGTTGGAGAGAATA	26	46.2	8108.3	66.4
2s	Cytb F degen		GGGTTGTCCTACTCCTTCTRG	21	54.8	6387.2	62.3
2s	Cytb R degen		GGRTTYGCYGGGGTGAAGTTATC	23	54.3	7158.7	66.2
2a	COI F Ntop	COI	TCTGATGATCGGGGCGCCTGAC	22	63.6	6767.4	70.0
2a	COI R Ntop		TGTGAGGTCTACAGATGCCCCCGCA	25	60.0	7643.0	72.4
2s	COI F M118		AGGCCTCGATCCTACAAACTTTTAGT	26	42.3	7905.2	65.9
2s	COI F M15		GTAATAATTTTCTTTATAGT	20	15.0	6111.0	47.1
2s	COI R M24		TGGTTATGTGSTTGGCTTGAAAC	23	43.5	7120.7	64.7
2s	COI R M18		GGGTCGAAGAATGTAGTGTT	20	45.0	6252.1	59.9
3s	ND2B-L	ND2	AAGCTTTCGGGCCCATACCC	20	60.0	6038.0	60.2
3s	ND2E-H		GGATTTTAGATCATGTGGTTGCAAGGGT	28	42.9	8729.7	59.3
4s	Dloop-Thr-F	D-loop	AGCACCGGTCTTGTAAACCG	20	55.0	6102.0	57.7
4s	Dloop-Phe-R		GGGCTCATCTTAACATCTTCA	21	42.9	6356.2	52.2

Notropis topeka Primer Development

After the tissue-derived DNA extractions were completed, we aligned the sequences to identify potential genetic markers and develop the potential quantitative primers for testing using SeAl 2.0 (Rambaut 2002), and BioEdit 7.1.9 software (Hall 1999). This was a completely novel endeavor. Neither traditional PCR nor quantitative PCR (qPCR) primers have been previously published for N. topeka.

The first marker was developed for COI since it has been a commonly used target gene for taxonomic investigations (Ward et al. 2009, Shokralla et al. 2011), and COI sequences for some co-occurring species were readily available. The second set of trial markers were developed for CytB, which is another gene region recently used for identification of aquatic macrofauna (Ficetola et al. 2008, Goldberg et al. 2011, Minamoto et al. 2012, Thomsen et al. 2012). Nucleotide differences between the *N. topeka* and *N. stramineus* mitochondrial gene sequences developed in this study were approximately 7% to 29% divergent overall with 1.1% to 2.4% of base pairs diagnostically different (i.e., pairwise differences at a particular locus between all sequenced *N. topeka* specimens and all sequenced *N. stramineus* specimens) (Table 4).

Primers were designed by inspection using 50 individuals from the 3 major populations of *Notropis topeka* (Michels 2000) as the target group, and a mix of co-occurring species as the out group (Table 5). The closest co-occurring relative, the sand shiner (*Notropis stramineus*) (Pittman 2011), was included in the out group. Primers were designed to be between 18 and 25 base pairs long with approximately 50% GC content, and approximately equal melting temperatures (per OligoAnalyzer 3.1, IDT, USA). In addition, primers were designed wherever possible to minimize dimers, hairpins, and other secondary structures, to contain multiple nucleotide differences, and to end with a characteristic G or C difference from the non-target sequence at the 3' end for forward primers and the 5' end for reverse primers (i.e., 3’ end of the reverse complement of the alignment sequence). Amplicons were selected to be roughly 75 to 200 base pairs in length. Prior to laboratory testing, potential primers were tested via BLAST search to confirm *Notropis topeka* specificity *in silico*.

Table 4. Mitochondrial nucleotide sequence differences between the target species (*Notropis topeka*) and the closest related non-target species (*Notropis stramineus*) as found in this study.
Sequence lengths are measured by number of base pairs (bp). The term sequenced difference denotes the condition where any one individual of *N. topeka* had a different base from any one individual of *N. stramineus* at a particular locus. Diagnostic difference denotes the condition where all *N. topeka* individuals had a different base than all *N. stramineus* individuals at a particular locus. Diagnostic differences are required for the assay to discriminate between the target and non-target species.

Gene	Sequenced Length (bp)	Sequenced Difference (bp)	% Sequenced Difference	Diagnostic Difference (bp)	% Diagnostic Difference
Cytochrome oxidase C subunit 1 (COI)	652	44	6.7	7	1.1
Cytochrome oxidase II subunit B (CytB)	418	54	12.9	9	2.2
NADH dehydrogenase 2 (ND2)	1035	298	28.8	14	1.4

Table 5. Mixture of tissue extracted DNA from non-target species used in primer efficacy trials.
Equal volumes (5uL) of each extract were combined to form a 27.8 ng/uL mixture, and 5uL of that mixture were loaded per reaction as template for the mixture trials.

Common Name	Species	KU Fish Tissue Catalog Number	Extract Concentration (ng/uL)	Amount Loaded Per Reaction (ng)
Topeka shiner	*Notropis topeka*	10153	10.5	8.75
Sand shiner	*Notropis stramineus*	2657	30.1	25.1
Central stoneroller	*Compostoma anomalum*	7642	43.1	35.9
Red shiner	*Cyprinella lutrensis*	2836	34.5	28.8
Mimic shiner	*Notropis volucellus*	8865	29.1	24.3
Bluntnose minnow	*Pimaphales notatus*	8054	19.6	16.3

Optimization of the PCR and qPCR conditions was found to be critical to distinguish between the two most closely related species (in this case between the endangered *N. topeka* and the ubiquitous sand shiner, *N. stramineus*). Within the PCR thermocycle, annealing temperature, salt concentration, and other factors have been shown to affect amplification of positive controls (Heid et al. 1996). PCR program development was performed using factorial experimental design with positive controls of *N. topeka*. A 2-

step thermocycle profile was optimized for conventional PCR, and a SYBR Green assay was optimized for qPCR. Once the PCR and qPCR programs were optimized, the primers were validated with positive and non-target controls. Primers were tested under various conditions (i.e., dispersed in pure water, various concentrations, mixtures, and spiked in environmental samples) using *N. topeka* tissue extracted positive controls, positive control spiked mixtures of tissue extracted target and non-target DNA, positive controls spiked in environmental samples, and both non-target (*N. stramineus*) and no template controls. For every trial all positive controls (including spiked mixtures and tissue extracts) successfully amplified the target amplicon, and all negative controls (non-target controls and no template controls) did not.

One PCR marker using COI and one using CytB were developed to reliably distinguish between *N. topeka* and its closest relative, *N. stramineus*, in endpoint assays. A third primer set using CytB was also able to amplify *N. topeka* but was difficult to distinguish between *N. topeka* and *N. stramineus* (Table 6). These same markers reliably amplify both *N. topeka* and *N. stramineus* in quantitative PCR assays using SYBR Green chemistry, but distinction between the two species is theoretically possible without sequencing through melt curve analysis. Peak melt curve temperatures were predicted for the COI and CytB amplicons with uMelt 2.0.2 software (Dwight et al. 2011), using the Weber thermodynamic set (Weber 2015) at a resolution of 0.5 °C, which is the same resolution as the iCycler instrument used for the sample analysis. A 50 mM monovalent ion concentration and a 3 mM free magnesium ion concentration were modeled, since the 2 x iQ SYBR Green Supermix used for the assay contains 100 mM KCl buffer and 6 mM MgCl2 (per BioRad, USA). Differences between melt curve peaks for *N. topeka* and *N. stramineus* were consistent with changes in G-C content of amplicons between the two species, with larger differences in percent G-C yielding larger differences in peak melt temperature (Table 6). Differences in both predicted and observed melt peak temperatures were largest for the CytB 498F/579R assay (Table 6, Figure 3). Both CytB assays showed promise in initial testing, but in dilution trials using positive controls (pure tissue-extracted *N. topeka* DNA), non-target controls (pure tissue-extracted *N. stramineus* DNA), no template controls (molecular grade water), and template DNA from a mixture of

species (Table 5), the larger amplicon of the 498F/678R assay was inconclusive (Figure 4), while the smaller 498F/579R assay was still able to successfully distinguish between the two species (Figure 5).

Table 6. Amplicon lengths with predicted and observed peak melt temperatures.
Predicted peak melt temperature was calculated via uMelt 2.0.2 (Dwight et al. 2011) using Weber (Dwight et al. 2015), 50 nM [Mono+], 3 mM free [Mg++], and 0% DMSO. Percent nucleotide difference refers to base pair differences between *Notropis topeka* and *Notropis stramineus* for the given amplicon.

Parameter	Species Category	Amplicon		
		COI F Ntop / COI R Ntop	**CytB 498F / 579R**	**CytB 498F / 678R**
Length (bp)	-	187	82	181
% Nucleotide Difference	-	4.8	7.3	5.5
G-C Content (%)	*N. topeka* *N. stramineus* Difference	55 52 3	55 50 5	54 50 4
Predicted Peak Melt Temperature (ºC)	*N. topeka* *N. stramineus* Difference	93 92 1	88.5 86.5 2	93 91.5 1.5
Observed Peak Melt Temperature (ºC)	*N. topeka* *N. stramineus* Difference	87.5 87.5 0	85.5 81.5 4	88 87.5 0.5

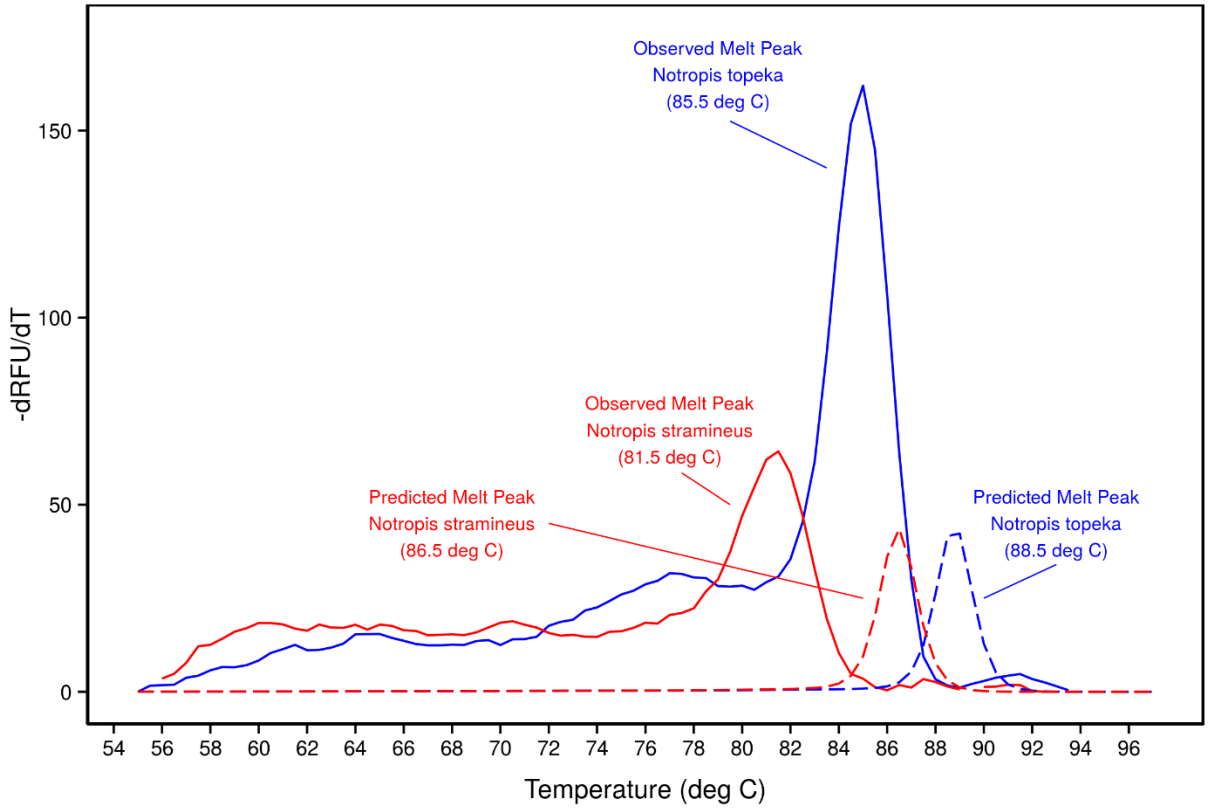


Figure 3. Comparison of observed and predicted melt curves for amplicons amplified by the Ntop CytB 498F / Ntop CytB 579R primer set.
Dashed lines represent the predicted results and solid lines represent the observed results. Predictions were made with uMelt 2.0.2 software (Dwight et al. 2011) using 50 mM monovalent ion concentration, 3 mM free divalent magnesium ion concentration, a 0.5 degree Celsius resolution, 0% DMSO, and the thermodynamic set from (Weber 2015).

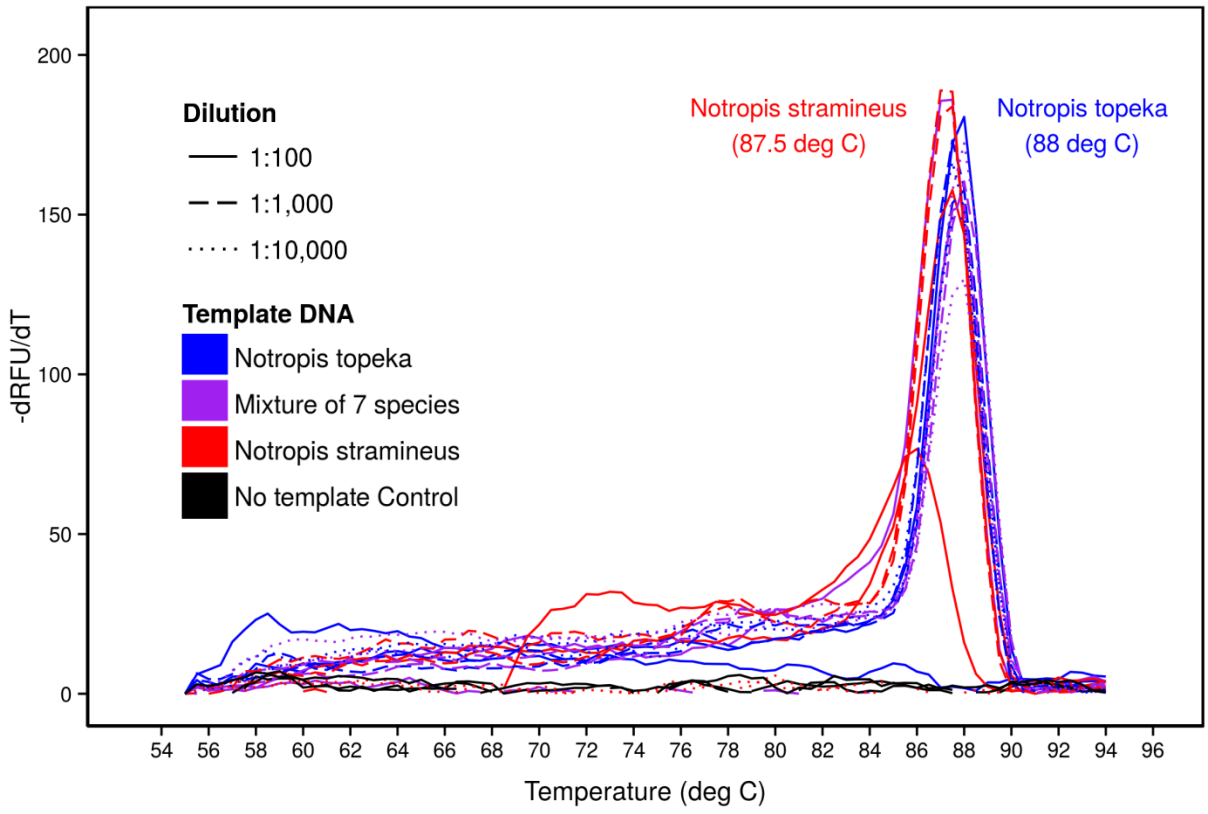


Figure 4. Melt curve analysis for amplicons amplified by the Ntop CytB 498F / Ntop CytB 678R primer set.
Template DNA sources were *N. topeka* tissue extracted DNA (50.5 ng), *N.stramineus* tissue extracted DNA (150 ng), and a mixture of tissue extracted DNA from multiple closely related and co-occurring species including both *N. topeka* and *N. stramineus* (see text for more details on mixture preparation). Serial dilutions of tissue extracts ranged from 1:1,000 to 1:100,000. The no template control was molecular grade sterile water.

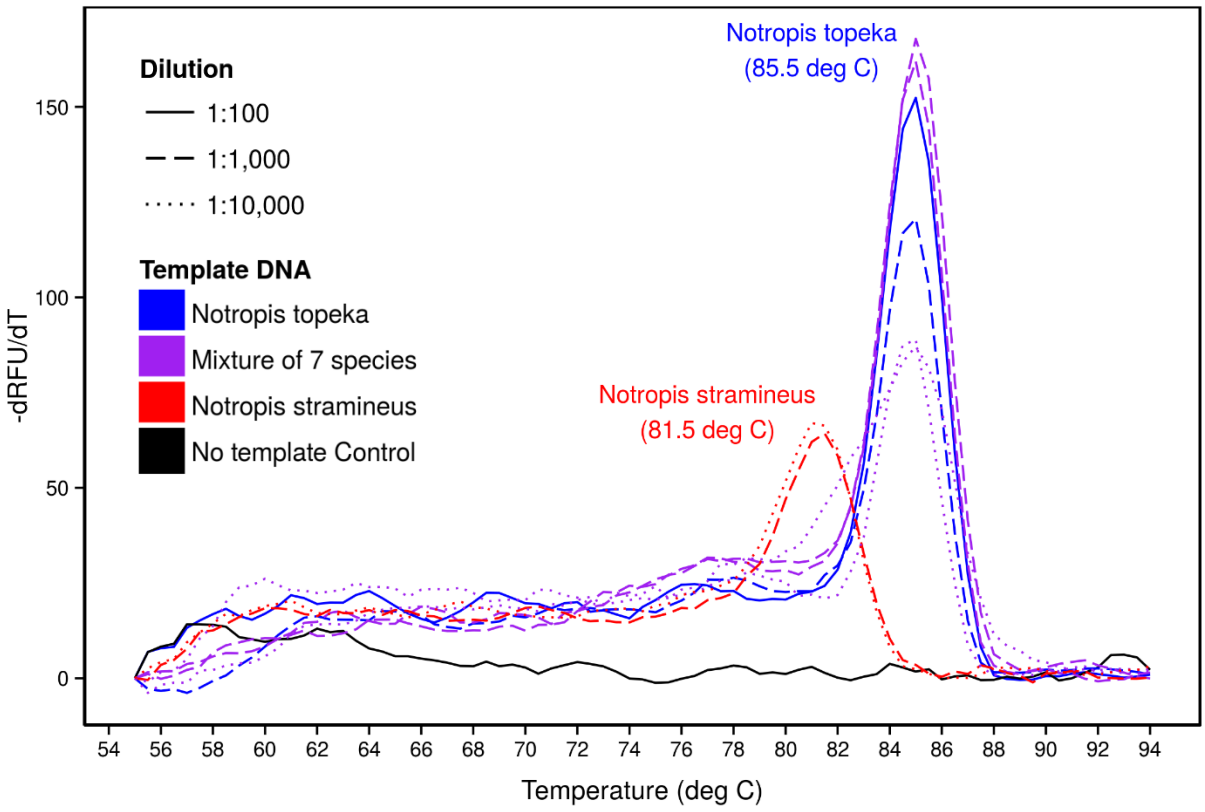


Figure 5. Melt curve analysis for amplicons amplified by the Ntop CytB 495F / Ntop CytB 579R primer set.
Template DNA sources were *N. topeka* tissue extracted DNA (50.5 ng), *N.stramineus* tissue extracted DNA (150 ng), and a mixture of tissue extracted DNA from multiple closely related and co-occurring species including both *N. topeka* and *N. stramineus* (see text for more details on mixture preparation). Dilutions of tissue extracts ranged from 1:1,000 to 1:100,000. The no template control was molecular grade sterile water.

Tissue Sample Testing

PCR markers were tested through a series of tissue extracted DNA trials. First, thermal gradient PCR was run to optimize annealing temperatures for the COI marker (Table 7) and the two CytB markers (Table 8, Table 9) using *N. topeka* tissue extracted DNA as a positive control, *N. stramineus* tissue extracted DNA as a non-target control, and sterile water as a no template / no amplification control (NTC). Tested thermal ranges spanned from at least 5 degrees below the lower calculated melting temperature of the primer pair to at least 5 degrees above the higher calculated melting temperature of the primer pair (50 ºC to 92 ºC for COI and 60 ºC to 80 ºC for both CytB primer pairs). Initial PCR conditions were 94 ºC for 2 min followed by 35 cycles of 95 ºC for 1 minute and the thermal test range for 30 sec. Samples were held on the thermocycler at 12 ºC for immediate post PCR processing and agarose gel electrophoresis.

Table 7. Thermal gradient testing results for primer pair COI F Ntop / COI R Ntop.
Templates were tissue extracted DNA from KU Natural History Museum collection specimens *Notropis topeka* 10187 and *Notropis stramineus* 2657. Positive amplification is denoted by a plus sign, while no amplification is denoted by a dash. Diagnostic annealing temperatures (i.e., the highest annealing temperature where *N. topeka* amplifies, but *N. stramineus* does not) are indicated by an asterisk.

Template DNA	Concentration (ng)	Annealing Temperature (ºC)															
		50	51	53	55.9	59.5	62.5	64.1	65	72	73.4	76.1	79.9	84.7	88.7	90.9	92
Notropis topeka	18.9×10^{-4}	+	+	+	+	+	+	+	+	+*	+*	-	-	-	-	-	-
Notropis stramineus	30.1×10^{-4}	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-

Table 8. Thermal gradient and concentration testing results for primer pair NtopCytB498F / NtopCytB579R.
Templates were tissue extracted DNA from KU Natural History Museum collection specimens *Notropis topeka* 10187 and *Notropis stramineus* 2657. Positive amplification is denoted by a plus sign, while no amplification is denoted by a dash. Diagnostic annealing temperatures (i.e., the highest annealing temperature where *N. topeka* amplifies, but *N. stramineus* does not) are indicated by an asterisk.

Species	Concentration (ng/uL)	Annealing Temperature (ºC)							
		61.6	63.4	65.6	68.2	70.9	73.6	76	78.1
Notropis topeka	10.5×10^{-2}	+	+	+	+*	+*	-	-	-
Notropis topeka	10.5×10^{-3}	+	+	+	+*	+*	-	-	-
Notropis topeka	10.5×10^{-4}	+	+	+	+*	+*	-	-	-
Notropis stramineus	30.1×10^{-3}	-	-	-	-	-	-	-	-
Notropis stramineus	30.1×10^{-4}	-	-	-	-	-	-	-	-

Table 9. Thermal gradient testing results for primer pair NtopCytB498F / NtopCytB678R.
Templates were tissue extracted DNA from KU Natural History Museum collection specimens *Notropis topeka* 10187 and *Notropis stramineus* 2657. Positive amplification is denoted by a plus sign, while no amplification is denoted by a dash. Diagnostic annealing temperatures (i.e., the highest annealing temperature where *N. topeka* amplifies, but *N. stramineus* does not) are indicated by an asterisk.

Species	**Concentration**	**Annealing Temperature (ºC)**							
	(ng/uL)	**61.6**	**63.4**	**65.6**	**68.2**	**70.9**	**73.6**	**76**	**78.1**
Notropis topeka	10.5×10^{-4}	+	+*	+*	-	-	-	-	-
Notropis stramineus	30.1×10^{-4}	-	-	-	-	-	-	-	-

Based on positive amplification results, the thermocycle was adjusted to a two-step process to optimize both discrimination between *N. topeka* and *N. stramineus* and time between temperature cycling. Annealing temperatures were selected to be as high as possible while still allowing for positive amplification with selective annealing of the primers to the target. Sensitivity trials were then carried out to evaluate the ability of the markers to discriminate between different conditions. Both positive controls (*N. topeka* tissue extracted DNA) and non-target controls (*N. stramineus* tissue extracted DNA) were serially diluted from full strength (31.5 ng/uL and 30.1 ng/uL, respectively) to 1:1,000,000 to determine preliminary detection limits for the COI primer pair (Table 10). Similar trials were performed for the CytB primer pairs using lower positive control concentrations (10.5 ng/uL from 1:1 to 1:10,000).

A test mixture of tissue extracted DNA from co-occurring species was also prepared from 5 commonly co-occurring, non-target species spiked with *N. topeka* tissue extracted DNA (Table 5). This mixture was also serially diluted. PCR testing for the COI and CytB markers showed the ability to positively detect *N. topeka* DNA from tissue extracts in both the positive controls and the mixtures without amplifying either the non-target controls or the no template controls across a broad range of concentrations (Figure 5). Initial testing of serial dilutions of tissue extracted DNA by conventional PCR suggested positive detection with COI markers as low as 1.57×10^{-10} ng per reaction (Table 10).

Table 10. Gel visualization results from initial limit of detection (LOD) testing.
Conventional PCR assay performed using the COI F Ntop / COI R Ntop primer pair and intial tissue extracted DNA from KU specimen 10190, with initial extract concentration measured by Nanodrop spectrophotometer. Positive ampification was determined by gel electrophoresis and is indicated by a plus symbol. Failure to amplify is denoted by a dash. The no template control did not amplify.

Initial Concentration (ng/reaction)	**Dilution Series**								
	10^{-2}	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**	**10^{-10}**
157.5	+	+	+	+	+	+	+	-	-

Quantitative PCR trials were also carried out using the COI and CytB markers and SYBR Green chemistry (BioRad Technologies, USA) with melt curve analysis. SYBR Green chemistry was chosen: (1) to use previously confirmed working primers without modification; (2) to provide quantitative capability with melt curve validation of PCR products; and (3) to allow for development and testing of additional primer sets at relatively low cost. Melt curve analysis has been shown to effectively identify specific PCR products (Ririe et al. 1997), and melt peak temperature prediction, gel electrophoresis, and sequencing were used for verification of PCR products for a subset of samples. Thermal gradient and serial dilution testing of the positive controls (*N. topeka*), mixture, non-target controls (*N. stramineus*), and no template controls (water) confirmed the findings from conventional PCR. In addition, melt curve analysis showed the potential for discrimination between *N. topeka* and *N. stramineus* amplicons using the CytB 498F / 579R primer set (Figure 5).

Environmental Matrix Testing

In addition to testing in pure water, amplification of tissue extracts was tested in environmental samples. Environmental samples were collected from waterbodies containing no fish, sunfish only (*Lepomis humilis and Lepomis cyanellus*), mixed cultures (*N. topeka* and *Lepomis spp.*), and pure cultures of *N. topeka*. eDNA was collected by both filtration (e.g., Jerde et al. 2011) and precipitation (e.g., Ficetola et al. 2008), then extracted by one of four methods: using the tissue extraction method outlined above, by commercially available kits (PowerSoil and PowerWater, MoBio, USA), by phenol-chloroform (e.g., Hillis et al. 1996), and by CTAB and chloroform (e.g., Turner et al. 2014). Positive controls amplified consistently for both filtration and precipitation collections and for all four extractions. In addition, non-target and no template controls did not amplify, and positive control spikes (0.10 ng / uL tissue-extracted *N. topeka* DNA) were detected in environmental samples, suggesting inhibition was not a limiting factor for detection in surface water samples taken from ponds and tanks at the University of Kansas field station.

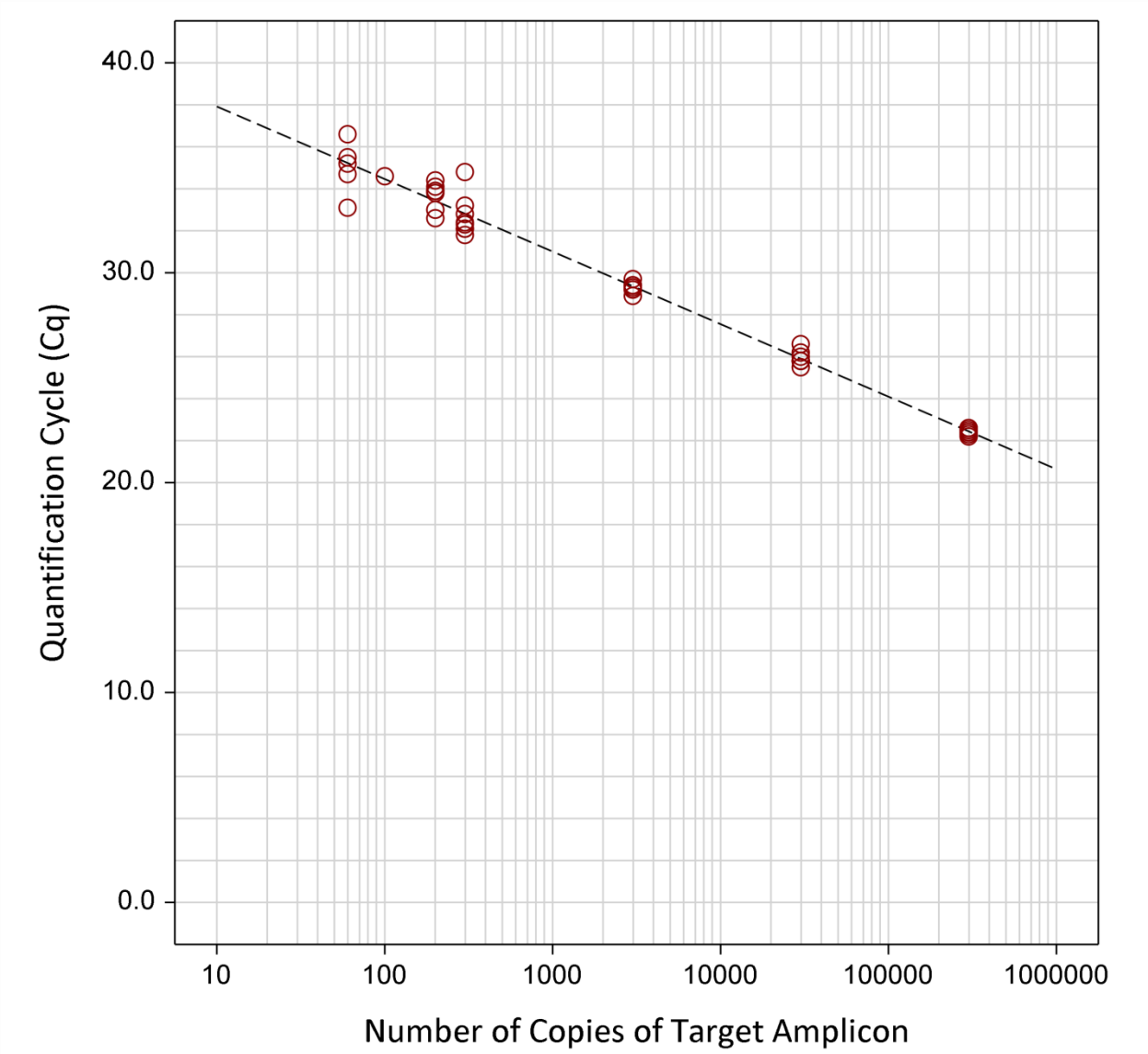
Standard Curve Development for Quantitative Assays

Two regions (1928 bp in COI and 260 bp in CytB) were amplified using previously published primers known to amplify specific sections of mitochondrial DNA in other fishes (Table 3). For each gene, the resulting amplicons were confirmed by agarose gel visualization, then quantified using Qubit dsDNA High Sensitivity assay (Life Technologies, Inc., USA). Full length clones were constructed using the pCR4-TOPO vector in TOPO-TA plasmid cloning kits (Life Technologies, Inc., USA) for insertion into TOPO-TA One Shot competent cells (Life Technologies, Inc., USA). Cells were plated on LB media selective plates (50 ug/mL kanamycin for treatments and 100 ug/mL ampicillin for plasmid controls) per manufacturer's recommendations and cultured overnight. Single colonies were then selected at random for testing and transferred to liquid media for batch culture. Plasmids were extracted from the randomly selected colonies using Pure Link Quick Plasmid Miniprep Kit (Life Technologies, Inc.) per manufacturer's recommendations, including the optional wash solution step and elution in 100 uL of TE buffer. Plasmid DNA concentrations were measured using the Qubit dsDNA Broad Range Assay (Life Technologies, Inc., USA), and transformation was verified by conventional PCR with subsequent agarose gel visualization. For those colonies with successful transformations, two were selected for batch culture, quantified via Qubit dsDNA Broad Range Assay, then serially diluted to produce a standard curve ranging from 3×10^6 copies per uL to 3 copies per 10uL.

Detection Limits for Quantitative Assays

In initial detection limit testing, serial dilutions of known concentrations spanning $3x10^6$ copies per uL to 3 copies per uL were prepared and used as templates in duplicate testing for the COI F Ntop / COI R Ntop and Ntop CytB 498F / Ntop CytB 579R primer sets. Additional detection level testing was focused on the latter, since melt peak analysis for this primer pair was able to discriminate between *N. topeka* and *N. stramineus*. An assay trial was run in octuplicate for a range of concentrations (Figure 6, Table 11) to determine assay efficiency and detection limits. The lower limit of detection for the Ntop

CytB 498F / CytB 579R primer set was determined to be 32 copies per reaction (American Public Health Association et al. 2012). Quantification limits were calculated as 5 times the lower level of detection, 160 copies per reaction (American Public Health Association et al. 2012).



Y = 41.329 - 3.442 X ; Efficiency = 95%, R2 = 0.98

Figure 6. Standard curve for Ntop CytB 498F / 579R assay based on cloned plasmid standards. Each target eDNA concentration was run in octuplicate.

Table 11. Lower limit of detection (LLD) and limit of quantification (LOQ) trials for Ntop CytB 498F / Ntop CytB 579R quantitative polymerase chain reaction (qPCR) assay for Notrops topeka.
Bold indicates lowest nominal copy number with at least 99% detection. Standard dilutions of the plasmid-cloned portion of CytB from *Notropis topeka* were used as template. See text for details on plasmid cloning.

Nominal Number of Copies	Number of Replicates	Number of Positives	Mean of Calculated Copies	Standard Deviation of Calculated Copies	t-value (n-1, 0.02)	Lower Level of Detection (copies) (tvalue*std dev)	Level of Quantification (copies) (5 * LLD)
300000	8	8	2.550×10^{10}	1.41	2.998	4.3	21
30000	8	8	29400	2.16	2.9985	6.5	32
3000	8	8	3080	1.70	2.998	5.1	25
300	**8**	**8**	**323**	**9.12**	**2.998**	**27**	**137**
200	8	7	167	4.33	3.143	14	68
100	8	1	89.9	1.00			
60	8	5	67.9	19.0	3.747	71	356
30	8	0					
15	8	0					
9	8	0					

CONCLUSIONS

In this study we were able to extract DNA from tissue samples of 185 vouchered individuals of the endangered fish, Topeka shiner (*Notropis topeka*) and sequence four gene regions, cytochrome oxidase 1, cytochrome oxidase b, NADH dehydrogenase 2, and the mitochondrial D-loop. Prior to this study, less than 10 sequences were available in GenBank for this species. Similar extractions and sequencing were also carried out for 10 sand shiner individuals (*Notropis stramineus*) and individuals of 9 other co-occurring species. In addition, we were able to design multiple primer sets for amplification of *Notropis topeka* DNA from laboratory and environmental samples that were able to distinguish *N. topeka* from *N. stramineus* under certain conditions. These tissue extractions, sequence data, and primer sets broaden the foundation of knowledge for genetic monitoring and conservation of the federally-listed, endangered Topeka shiner.

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CHAPTER 3: Environmental DNA (eDNA) as a potential indicator of presence and abundance in aquatic systems: an extended mesocosm study using an endangered fish (Topeka Shiner, *Notropis topeka)*

INTRODUCTION

Since every organism has DNA, and since that DNA is shed into the environment, there is the potential for recovery of DNA from environmental samples. However, the relationship between the amount of DNA released from the organism, the amount of DNA recoverable by the investigator, and the original biomass or number of organisms is still largely unknown. Two recent studies (Takahara et al. 2012, Thomsen et al. 2012) have suggested that the amount of fish DNA present in the water column of aquatic systems may be related to the number of fish present. Given the federal mandate to monitor populations of endangered species (Endangered Species Act 1973), we developed an experiment to investigate the potential use of environmental DNA as an indicator for monitoring the federally listed, endangered Topeka shiner (*Notropis topeka*) based on either the detection/nondetection or quantity of *N. topeka* DNA recovered from aquatic mesocosms.

We hypothesized that *Notropis topeka* DNA in the water column would increase over time to a stable, recoverable, and measurable background concentration. This increase was hypothesized to have an initial exponential accumulation phase, similar to logistic growth models in batch reactors, based on the idea that production rates would increase with growth of fish (as recently described by Klymus et al. (2015), but would reach some maximum value where losses to degradation and partitioning would balance production. As an extension of this hypothesis, we believed that higher numbers of fish would both reach a higher background concentration and more rapidly reach that concentration than lower numbers of fish (Figure 1). Positive detections of *Notropis topeka* were expected to follow a similar pattern.

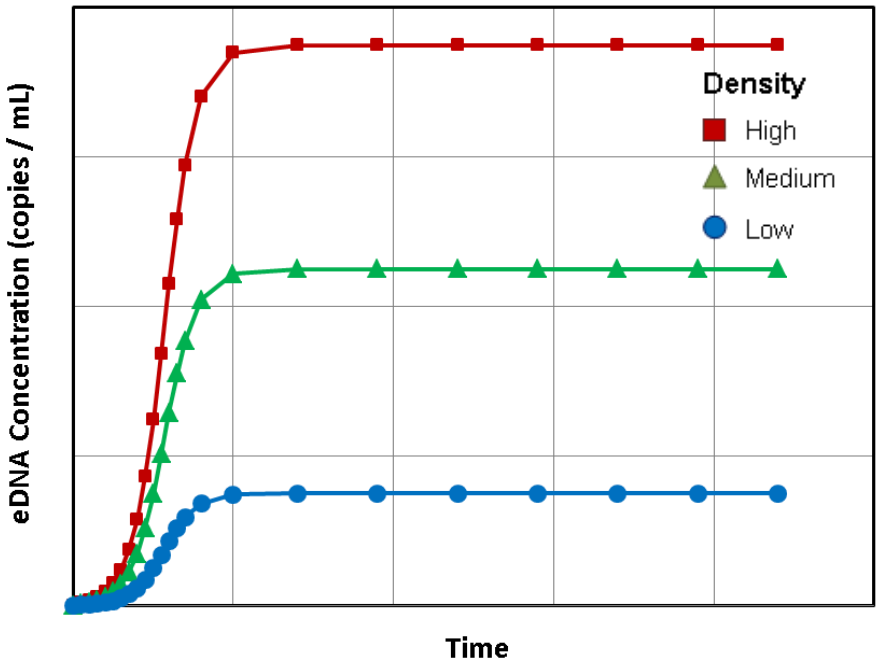

Figure 1. Predicted increase in eDNA concentration with time.
Red squares, green triangles, and blue circles represent high, medium, and low density treatments (i.e., high, medium, and low counts of fish in identical volumes of water), respectively. Curves are based on logistic growth ($A / (1 + B e^{-kt})$) with identical rate parameters (B, k), but with upper limit parameters (A) that increase with increasing count of fish.

METHODS

Overview

Topeka shiners have been in continuous culture at the KU Field Station (KUFS) since 2002, when 291 individuals were relocated from Deep Creek and Willow Creek in Kansas. Fish from this culture were stocked in three densities - high (80 fish), medium (40 fish), and low (20 fish) - in 10 m^3 tank mesocosms at KUFS. Two tanks were stocked at each density (Table 1). These tanks all reside in the same experimental unit, and the area surrounding the tanks was partially flooded to provide thermal mass and to help maintain thermal continuity among the tanks. The tanks were filled prior to stocking using a

single, aged well-water source, and primary water quality parameters were measured within each tank. Fish were weighed and measured prior to stocking and at the end of the experiment.

Water column samples were collected on a day, week, and month sampling scheme (Table 2) to observe potential accumulation and maintenance of eDNA in each tank. For each sampling event, five replicate integrated water samples were collected at in each tank. One field blank was also collected for each event. An additional water column sampling event was performed after removal of fish to observe eDNA signal loss once the source of the eDNA (i.e., the fish) was removed.

eDNA was extracted from water samples using cetyltrimethylammonium bromide (CTAB) extraction, chloroform separation, and precipitation of DNA using isopropanol and 5 M sodium chloride. Based on evidence of more accurate double stranded DNA quantitation methods than the Nanodrop spectrophotometer (Gallagher and Desjardins 2008), quantitation of eDNA extracts was performed using a Qubit spectrophotometer (Life Technologies, Inc.) using the Qubit dsDNA HS (double stranded DNA, high sensitivity) assay kit.

Because no *N. topeka* specific primers had been published, we developed two novel primer sets for *N. topeka* detection (see Chapter 2). This development required significant effort to extract DNA from tissues of vouchered specimens, sequence that DNA, align the sequences, design the primers, and test and optimize the primers for polymerase chain reaction (PCR) and quantitative PCR (qPCR). Since qPCR has a lower detection limit, higher resolution, and faster results than conventional PCR , detection information was developed primarily using qPCR methods. Similarly, the use of melt curve analysis of PCR products was used in lieu of gel electrophoresis for determination of specific amplicons, with target melt curves determined both using tissue-extracted template DNA from vouchered *N. topeka* specimens. Genetic sequencing of *N. topeka* and development of molecular methods for identifying and potentially describing populations of *N. topeka* not only provide the basis for the eDNA analyses proposed in this

work, but they also directly contribute to conservation efforts at the University of Kansas Field Station (KUFS) and the mandate for long-term monitoring and preservation of the species.

Table 1. Density treatments by tank.

Tank	Density	Initial Fish Count
1	High	80
2	Medium	40
3	Low	20
4	Low	20
5	High	80
6	Medium	40

Table 2. Sampling schedule for this project.

Sampling Date	Days Before Fish Stocking	Days After Fish Stocking	Days After Fish Removal
11/7/2013	1	0	
11/8/2013		1	
11/9/2013		2	
11/10/2013		3	
11/11/2013		4	
11/12/2013		5	
11/13/2013		6	
11/14/2013		7	
11/15/2013		8	
11/21/2013		14	
12/3/2013		26	
10/8/20014		335	
11/17/2014		375	27

Laboratory Methods

Great care was taken to prevent cross-contamination of laboratory samples. Before working with samples, between samples, and after working with samples, all work areas and equipment were cleaned using either DNA Away (Thermoscientific, Inc.) or chlorine bleach (10% sodium hypochlorite) and 10% ethanol. New gloves were used with each sample batch. Except where specifically noted otherwise, laboratory methods also followed recently suggested best practices for eDNA analysis (Goldberg et al. 2015). High-copy extractions (e.g. tissue) and low-copy extractions (e.g., environmental and separation of pre- and post-PCR processing of samples) were performed at different locations, samples were kept in long term storage at -80 °C, and the number of freeze/thaw cycles was minimized per recent recommendations (Takahara et al. 2015).

Tissue DNA extraction and sequencing

Using specimens currently held at the University of Kansas Natural History museum, we extracted DNA from vouchered tissue samples taken from 180 separate individuals of *Notropis topeka* and 26 individuals of 12 co-occurring species (Table 3). Tissue DNA was extracted with a standard digestion using protienase K and heat. DNA from a subset of the extractions was amplified for three mitochondrially encoded genes: cytochrome oxidase I (COI), cytochrome oxidase II subunit B (CytB), and NADH dehydrogenase 2 (ND2). The tissue extracted genes were cycle sequenced using an Applied Biosystems 3730 DNA Analyzer, and the sequences were aligned using SeAl 2.0 and BioEdit 7.1.9 software. Tissue extracts have been stored at -80 °C. Additional sequences used for *in silico* testing of our markers against COI, CytB, and ND2 were retrieved from GenBank.

eDNA Primer Development

After the tissue-derived DNA extractions were completed, we aligned the sequences to identify potential genetic markers and develop the potential quantitative primers for testing. This was a completely

novel endeavor. Neither traditional PCR nor quantitative PCR (qPCR) species-specific primers have been published for *N. topeka*.

Sequences for COI and CytB of both target (*N. topeka*) and non-target species (*N. stramineus, N. volucellus, Lepomis humilis,* etc.) were aligned using BioEdit 7.1.9 software. Base pair differences were found by examination, and primers were designed using the following guidelines: 18-25 base pair (bp) length primers with 75-150 bp amplicons; approximately 50% GC content with similar melting points for each primer in a pair; differences in base pairs at the 3' end of each new primer (both forward and reverse); and minimization of hairpins, primer dimers, and other undesirable structures. Potential primers were identified and tested for efficacy. Two primer sets, one for COI and one for CytB were found to reliably amplify *N. topeka* (Table 3). Optimization of both PCR and qPCR was performed by varying the annealing temperature and cycle length in the thermocycle protocol. Primers were tested under various conditions (e.g., DNA dispersed in pure water, mixtures of DNA in pure water, and mixtures of DNA at various concentrations) using *N. topeka* tissue extracted positive controls, positive control spiked mixtures of tissue extracted target and non-target DNA, positive controls spiked in environmental samples, and both negative (*N. stramineus*) and no template controls. A 2-step thermocycle profile was developed for the qPCR SYBR Green endpoint assays for both COI and CytB. The NtopCytB 498F / 579R assay was selected for qPCR analysis in this experiment, since it had been shown to be the more diagnostic of the two primer sets. However, because the experiments in this work were designed using only morphologically identified *N. topeka* descended from vouchered specimens in controlled conditions, distinction between the two species was not necessary in this case.

Table 3. Primer sets developed for and used in this study.

Mitochondrial Gene	Primer Name	Primer Sequence	Amplicon Length
Cytochrome Oxidase I (COI)	COIF Ntop	5'-TCTgATgATCggggCgCCTgAC-3'	187
	COI R Ntop	5'-TgTgAggTCAgATgCCCCCgCA-3'	
Cytochrome Oxidase II Subunit B (CytB)	NtopCytB 498F	5'-AggCTTTTCggTggATAACgCgACg-3'	82
	NtopCytB 579R	5'- CgTTgCACCggCAATgACgAAC-3'	

eDNA extraction

These methods involve surface water collection, followed by a modified cetyltrimethylammonium bromide (CTAB) extraction using chloroform separation and precipitation of DNA using isopropanol and 5M sodium chloride (e.g., Coyne et al. 2005, Ficetola et al. 2008, Turner et al. 2014).

15 mL water samples were collected then held at -20 °C in 33.5 mL of 95% ethanol and 1.5 mL of 3M sodium acetate in 50 mL sterile centrifuge tubes. The 50 mL tubes were spun at 3400xg for 35 minutes, then the liquid was decanted and the pellet retained. After 5 minutes of drying, 700 uL of CTAB were added, the samples were briefly vortexed, and then placed in a 60 °C oven for 10 minutes. After removal from the oven, the samples were spun at 3400xg for 5 minutes, and the full contents of each tube were transferred to low-bind centrifuge tubes containing 700 uL of chloroform. Samples were shaken horizontally for 5 minutes, then centrifuged at 15,000xg to separate the phases. 500 uL of each supernatant were transferred to new sterile tubes, then 500 uL of isopropanol and 250 uL of 5 M NaCl were added. Samples were held at -20 °C overnight to precipitate the extracted DNA. The next day, the samples were centrifuged at 15,000 x *g* for 5 minutes, then the liquid was decanted and the pellet retained. The pellet was then washed twice with 150 uL of 70% ethanol, spun at 15,000xg for 5 minutes, the liquid decanted, and the pellet again retained. After the second wash and decant, the sample tubes were placed

on their side in a UV sterilized and ethanol cleaned laminar flow hood for removal of any remaining ethanol. The last drops of ethanol were removed using sterile cotton swabs, being careful to avoid the pellet. Once the ethanol was evaporated, the pellets were eluted overnight at 4 °C in 100 uL of 10 mM Tris HCl buffer (pH 8.0) and 0.1 mM EDTA. DNA in the samples was quantified using a Qubit ds DNA high sensitivity assay, then subsequently split into aliquots for qPCR analysis (-20 °C) and long term storage (-80 °C).

Quantitative Polymerase Chain Reaction (qPCR)

Pre- and post-PCR sample handling were kept separate, with extraction and quantitation, sample storage, and qPCR all performed in separate laboratories. Prior to qPCR plate setup, all tubes, tube holders, well plates, and molecular grade water were sterilized for 15 minutes in a CL 1000 UV crosslinker (UVP, Inc., USA) at 200 J/cm2. qPCR mastermixes were combined and plates setup in a dedicated PCR hood, with positive airflow, UV sterilization, and strictly no-template consumables, pipetters, and equipment. For the CytB qPCR endpoint assay, we used 25 uL reactions with 12.5 uL of IQ Supermix (BioRad, Inc.), forward and reverse primers at 625 nM, and 5 uL of template DNA from environmental extractions. Water made up the remaining volume. Template DNA was added in a low copy lab, and positive controls were added in the high copy lab immediately prior to qPCR amplification. Samples were run on a BioRad iCycler using SYBR Green chemistry followed by melt curve analysis. Each plate contained a positive control (*N. topeka* tissue extracted DNA) and a no template control. Tank samples were tested for inhibition prior to endpoint assay runs by spiking with positive *N. topeka* controls. We used a 2 step thermocycle for the CytB primer pair: 3 minute hot start at 95 °C, followed by 50 cycles of 95 °C for 15 seconds and 70 °C for 1 minute. The subsequent melt curve analysis ranged from 55 °C to 95 °C in 0.5 degree steps every 10 seconds. Melt curve interpretation was assisted by prediction of melt curve peaks using uMELT 2.0 software (Dwight et al. 2011).

Field Methods

Tank Preparation

Six 10 m^3 tanks were drained, power washed, and bleached (10% sodium hypochlorite) to remove any potentially confounding DNA from *N. topeka* or other sources. The tanks were allowed to dry in the sun for 3 days, then filled. Fill water was originally ground water that had been well aged in a surface reservoir that did not contain fish, but did contain established phytoplankton and zooplankton communities.

Fish Stocking

N. topeka were collected from holding ponds at the KU Field Station facility. Collection was performed by seine, and the fish were transferred via aerated buckets. Total length and weight was recorded for 50 randomly selected individuals, and the remaining fish were weighed in batches prior to stocking. Two of the six tanks were randomly assigned to each of 3 treatments: high density, medium density, and low density. High density tanks were randomly stocked with 80 fish, medium with 40 fish, and low with 20 fish. Low density tanks corresponded to typical school sizes that had been observed in the wild (Stark et al. 2002). The phytoplankton and zooplankton communities introduced to each tank in the initial fill water were the primary source of food for the fish during the experiment. *N. topeka* commonly feeds on insects, zooplankton, detritus and plant matter (Kerns and Bonneau 2002, Missouri Department of Conservation 2010), and previous studies at KUFS had shown that this food source was sufficient for maintaining *N. topeka* populations over long periods of time. Therefore, no additional feeding was necessary for the duration of the experiment.

eDNA Sampling

Samples were collected in 12 sampling events: one prior to stocking, 10 while fish were present, and 1 after fish had been removed. For each sampling event, five integrated depth samples were taken using dedicated samplers for each tank, yielding a total of 10 replicate samples for each treatment. Samples were collected, tightly sealed, then held on ice in the dark. New gloves were used for each tank, and sample bottles were rinsed using tap water, then sprayed with 10% bleach, then rinsed again. In addition, for each event a field blank (sample bottle containing tap water) was placed on ice in the cooler prior to the first sample being collected, and the field blank accompanied the samples throughout processing. After collection, each sample bottle was well mixed, and 15 mL of water was transferred to a 50mL centrifuge tube containing 33.5 mL of 95% ethanol and 1.5 mL of 3 M sodium acetate via sterile serological pipette (Ficetola et al. 2008). Before and after sample processing, and between each tank, the bench was sterilized using 10% bleach, and new gloves were donned. After every 15 mL sample and the field blank had been transferred, the centrifuge tubes were placed on ice in the dark and transferred to the laboratory to be held at -20 °C until extraction.

Water Quality Data Collection

Throughout the experiment, *in situ* water quality parameters (water temperature, pH, specific conductance, dissolved oxygen concentration) were collected using a Horiba U-10 or U-50 water quality meter (HORIBA Instruments, Inc.). Secchi depth and water depth were also recorded. Sampling events began one day prior to stocking (day 0) and continued concurrently with sampling events (days 1,2,3,4,5,6,7,8,14,26,335,and 375). Basic water quality parameters were measured both to monitor potential tank, treatment, or environmental effects on eDNA concentration and to ensure that the fish did not experience stress from poor water quality. Sampling equipment was decontaminated in 10% bleach followed by tap water rinse between tanks to minimize cross-contamination.

Fish Removal

At the end of the experiment, the *N. topeka* were removed from the tanks by seine. Tanks were observed over several days to ensure removal of all visible individuals. Each fish was individually measured for total length, girth, and total weight, then placed in aerated buckets and returned to their ponds at KUFS.

Statistical Methods and Data Analysis

Water quality, population, and eDNA data were analyzed using NCSS 9 (Hintze 2013) and R 3.1.3 (R Core Team 2015) statistical software. Where appropriate, data were analyzed for significant differences by tank, then combined into groups by density treatment. Condition factor of the fish was calculated either using Fulton's K (K = 100,000 * total weight in g / (total length in mm)^3) or Richter's B' (B' = total weight in g / (total length in mm/10 * (girth in mm/10)^2)) (Richter et al. 2000).

Detection/Nondetection was determined by comparison of endpoint assay melt curve results with melt curves predicted by uMELT 2.0 software (Dwight et al. 2011) and melt curves of positive controls. When melt curve peaks aligned with predictions and positive controls, assays were judged to have positive detection even if suppressed amplification (i.e., increased cycle times) indicated relatively low initial quantities of target DNA. Detection/nondetection of quantitative PCR assay results was determined by comparison with the minimum detection limits of the assay (American Public Health Association et al. 2012).

Concentrations of eDNA were determined by calculation using standard curves based on known concentrations of serially-diluted plasmids containing the target amplicon. Standard curves for all amplifications had efficiencies between 95% and 105% (double check range) and R2 values > 0.95. The minimum detection limit of the assay was determined to be 36 gene copies/mL. Concentrations for

values that did not amplify were replaced with half the detection limit for purposes of aggregate calculations (e.g., mean, standard error, etc.).

RESULTS AND DISCUSSION

Water Quality and Environmental Parameters

Conductivity, dissolved oxygen, pH, and water temperature varied by tank, but all parameters remained within normal ranges for small waterbodies in the home range of the Topeka shiner (Minckley and Cross 1959) (Table 4). Changes in tank parameter values were significantly related to the date of sampling ($p<0.001$), but few other meaningful differences were observed. Tanks were aligned north - south in a 2 x 3 array, with tank 1 at the southeast corner and tank 4 at the southwest corner. While small differences were observed in temperature among tanks (tanks 1 and 4 were approximately 0.75 degrees C higher than the other tanks), those differences were smaller than the diurnal temperature fluctuation of the water column, and no significant differences were found among tank water temperatures in 3 months of previous monitoring (data not shown). Dissolved oxygen ranged from 8.8 mg/L to 16.1 mg/L, and pH ranged from 6.72 to 9.42 throughout the study. Both ranges were acceptable for fish according to published USEPA nationally recommended water quality criteria for aquatic life support (US Environmental Protection Agency 2009). Water clarity was measured by secchi depth, and tanks were observed to be clear to the bottom for all tanks and all measurements, except for the 335 day measurement in tank 3, where the secchi depth was 70% of the water column. These clarity measurements suggest light penetration to the bottom of the water column was present during the day for all tanks throughout the majority of experiment.

Average daily air temperature ranged from -20 °C to +30 °C over the course of the experiment (Figure 2), with temperatures ranging from +15 °C to -15 °C over the first 26 days of the experiment. Average daily wind speed ranged from 0 to 16 mph throughout the experiment, with a small seasonal

variation trend of 5-8 mph (Figure 3). Although photosynthetically active radiation (PAR) above teh water surface increased significantly during the summer season, average daily values for all sampling events were within a 100 uE /m^2/S range (Figure 4).

Table 4. Observed water quality parameters during the study.

Parameter	Statistic	Tank					
		1	2	3	4	5	6
Conductivity (mS/cm)	Maximum	0.308	0.261	0.257	0.288	0.258	0.286
	Mean	0.255	0.250	0.242	0.269	0.248	0.2638
	Minimum	0.143	0.207	0.196	0.193	0.202	0.201
	Standard Deviation	0.0418	0.0182	0.0180	0.0326	0.0207	0.0276
	Count	12	12	12	12	12	12
Dissolved Oxygen (mg/L)	Maximum	15.3	13.1	16.1	15.8	13.9	13.5
	Mean	11.8	12.0	12.2	12.1	11.8	11.8
	Minimum	9.5	8.8	9.5	9.3	9.5	9.2
	Standard Deviation	1.32	1.09	1.47	1.48	1.03	0.99
	Count	12	12	12	12	12	12
pH	Maximum	9.42	9.13	9.18	9.03	9.34	9.05
	Mean	8.59	8.62	8.53	8.49	8.60	8.53
	Minimum	8.18	7.74	6.72	8.14	8.12	7.51
	Standard Deviation	0.308	0.334	0.628	0.223	0.328	0.377
	Count	12	12	12	12	12	12
Water Temperature (degrees C)	Maximum	19.3	18.8	18.5	19.2	18.8	18.8
	Mean	9.1	8.4	8.5	9.1	8.4	8.5
	Minimum	3.54	2.77	2.87	3.69	2.99	2.23
	Standard Deviation	3.84	3.91	3.81	3.77	3.91	4.01
	Count	12	12	12	12	12	12

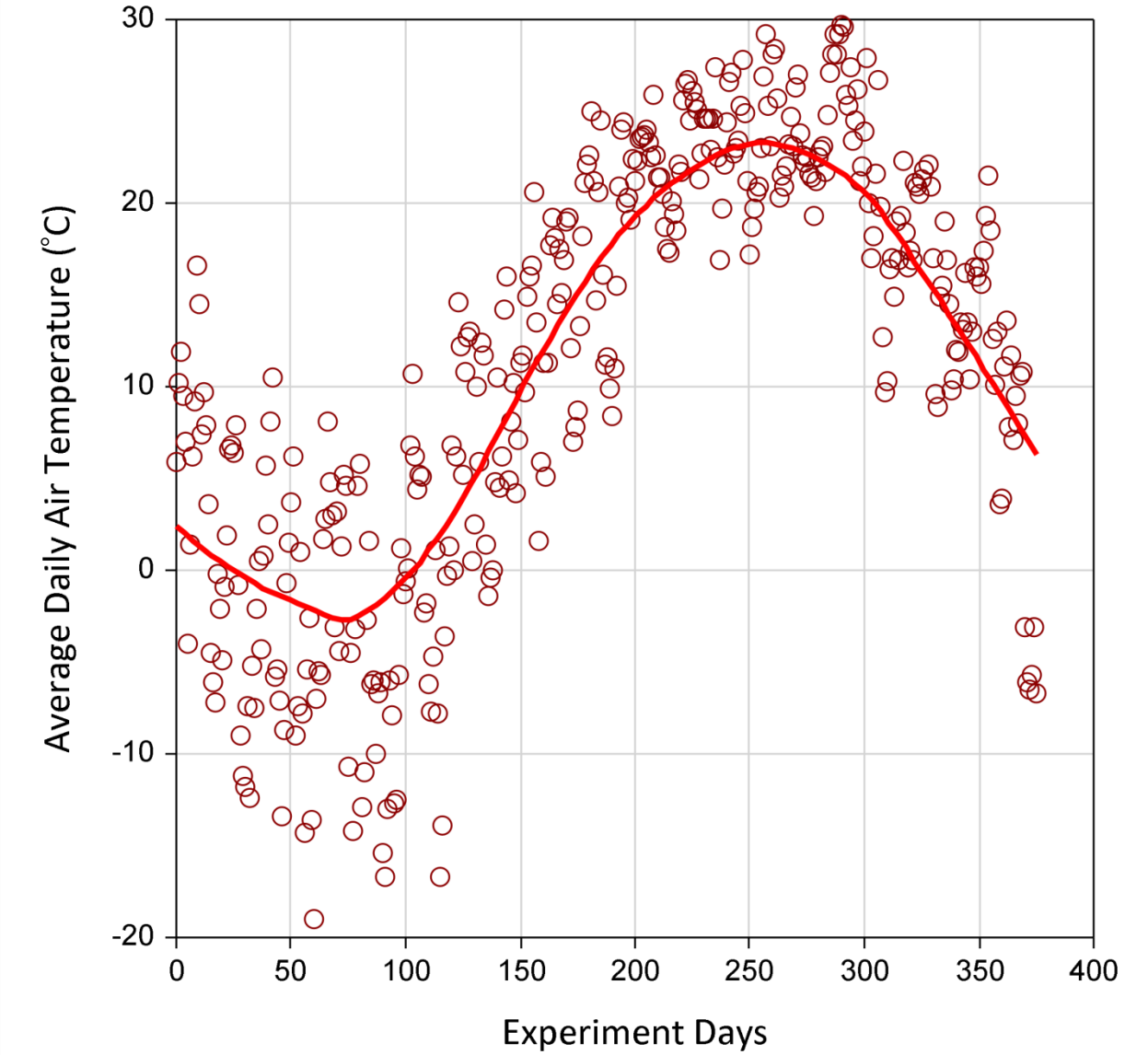

Figure 2. Average daily air temperature for the duration of the experiment.
A locally weighted smoothing regression (i.e., LOESS) curve was added to show the general trend observed over time. Data were collected by a weather station at KUFS located within 300 m of the experimental tanks (http://kufs.ku.edu/resources/weather-station).

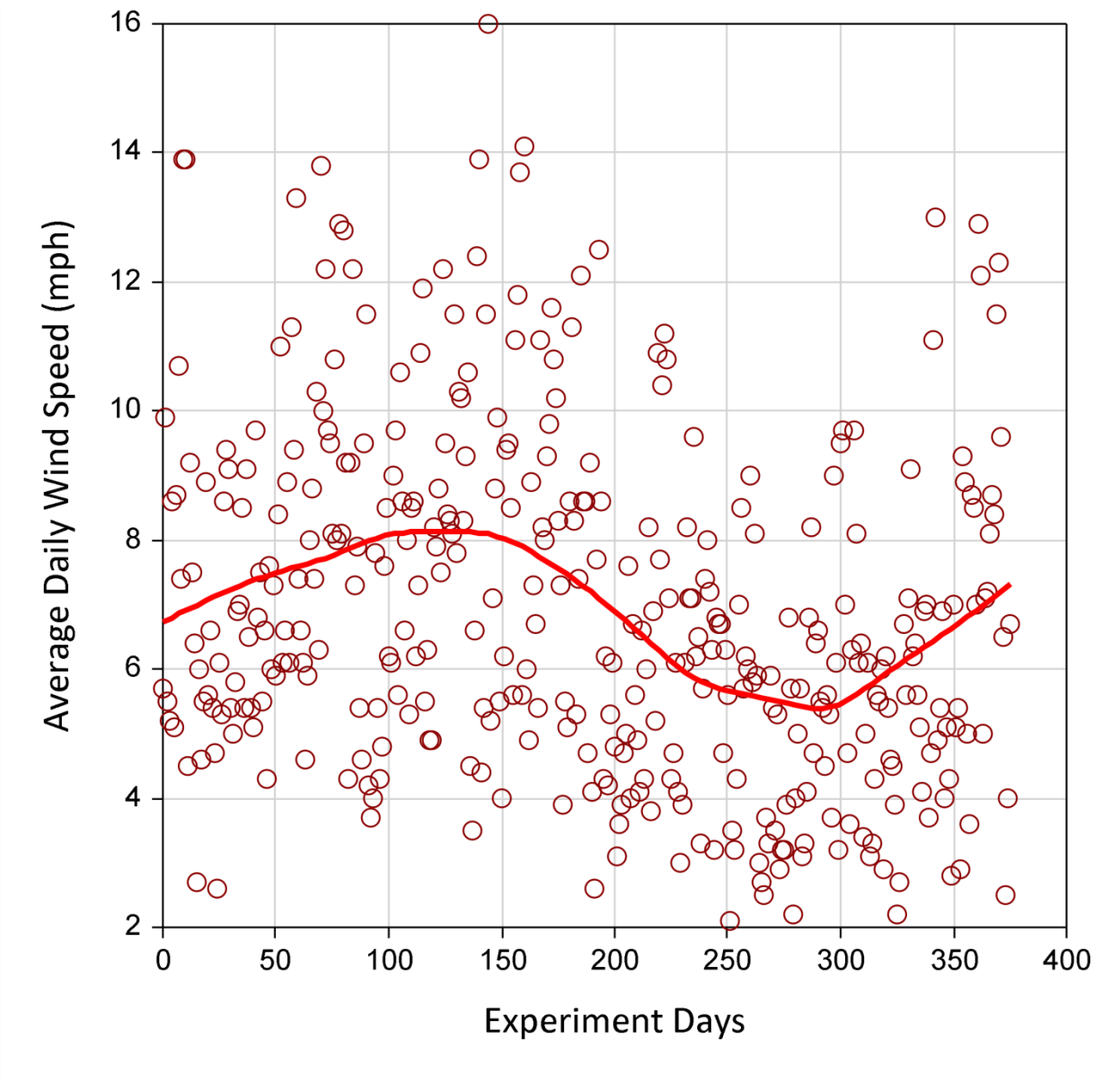

Figure 3. Average daily wind speed for the duration of the experiment.
A locally weighted smoothing regression (i.e., LOESS) curve was added to show the general trend observed over time. Data were collected by a weather station at KUFS located within 300 m of the experimental tanks (http://kufs.ku.edu/resources/weather-station).

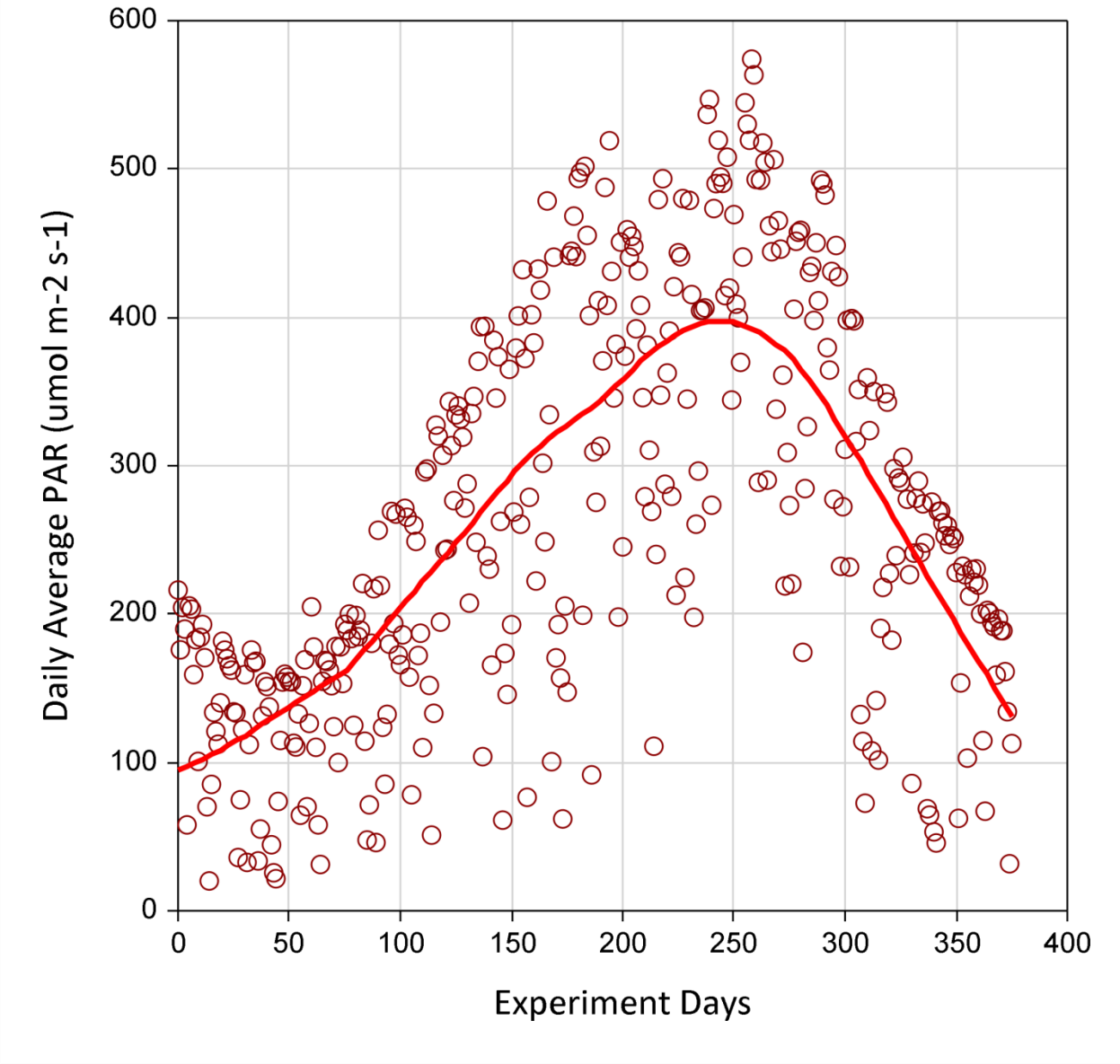

Figure 4. Average daily photosynthetically active radiation (PAR) levels for the duration of the experiment.
A locally weighted smoothing regression (i.e., LOESS) curve was added to show the general trend observed over time. Data were collected by a weather station at KUFS located within 300 m of the experimental tanks (http://kufs.ku.edu/resources/weather-station).

Fish Survival, Growth, and Condition

Despite the relatively short (2-3 years) expected lifespan of N.topeka (Stark et al. 2002), survival of Topeka shiners over the course of the 335 day experiment was high, with 16% or less mortality in all tanks (Table 5). High density tanks (initially stocked with 80 individuals) had the highest mortality, followed by low density tanks (20 individuals stocked) and medium density tanks (40 individuals stocked). Since schools of 20 - 30 individuals have been commonly observed for Topeka shiners in the wild (Stark et al. 2002), we expected the lowest mortality rates in these naturally occurring densities with higher mortality rates expected for higher density treatments (i.e., high and medium densities). However, losses in the medium density treatment were similar to the low density treatment.

Mean fish weights were calculated for each tank both during initial stocking and after fish removal (Table 6). Total length and girth measurements were also taken during fish removal (Table 6), and mean weight gain (Table 6, Figure 5), length to weight relationships (Figure 6), and condition factor (Table 6) were calculated for each tank and density treatment.

Table 5. Fish counts and mortality by tank and density treatment.

Parameter	High Density		Medium Density		Low Density	
	Tank 1	Tank 5	Tank 2	Tank 6	Tank 3	Tank 4
Count at Stocking	80	80	40	40	20	20
Count at Removal	70	67	40	39	18	19
Mortality	10	13	0	1	2	1
Percent Mortality	13%	16%	0%	<1%	10%	5%

Table 6. Fish growth and condition statistics by tank and density treatment.
Where available, standard deviations of statistics are shown in parentheses.

Sample	Parameter	High Density		Medium Density		Low Density	
		Tank 1	Tank 5	Tank 2	Tank 6	Tank 3	Tank 4
At Stocking	Total Weight (g)	126	114	52.6	61.5	36.2	35.2
	Average Weight (g)	1.57 (0.48)	1.43 (0.55)	1.31	1.54	1.81	1.76
	Average Total Length (mm)	57* (4.7)	55* (5.2)				
	Average Girth (mm)		10* (1.6)				
	Average Fulton's K**	0.85* (0.11)	0.83* (0.12)				
	Average Richter's B**		0.239* (0.030)				
At Removal	Total Weight (g)	122	105	73.2	75.1	54.2	51.4
	Average Weight (g)	1.75 (0.29)	1.58 (0.32)	1.83 (0.51)	1.92 (0.50)	3.01 (0.95)	2.7 (0.89)
	Average Total Length (mm)	59 (3.3)	58 (4.0)	60 (4.7)	61 (5.7)	68 (6.8)	66 (4.9)
	Average Girth (mm)	12 (1.4)	11 (1.5)	13 (1.4)	12 (1.5)	16 (2.7)	14 (3.0)
	Average Fulton's K**	0.839 (0.10)	0.806 (0.081)	0.823 (0.095)	0.837 (0.088)	0.908 (0.088)	0.912 (0.15)
	Average Richter's B**	0.222 (0.040)	0.214 (0.041)	0.194 (0.040)	0.22 (0.038)	0.174 (0.026)	0.205 (0.048)

* Measurements based on a subset of 50 individuals.

** Fulton's K = 100,000 * total weight in g / (total length in mm)^3)

*** Richter's B' = total weight in g / (total length in mm/10 * (girth in mm/10)^2)) (Richter et al. 2000)

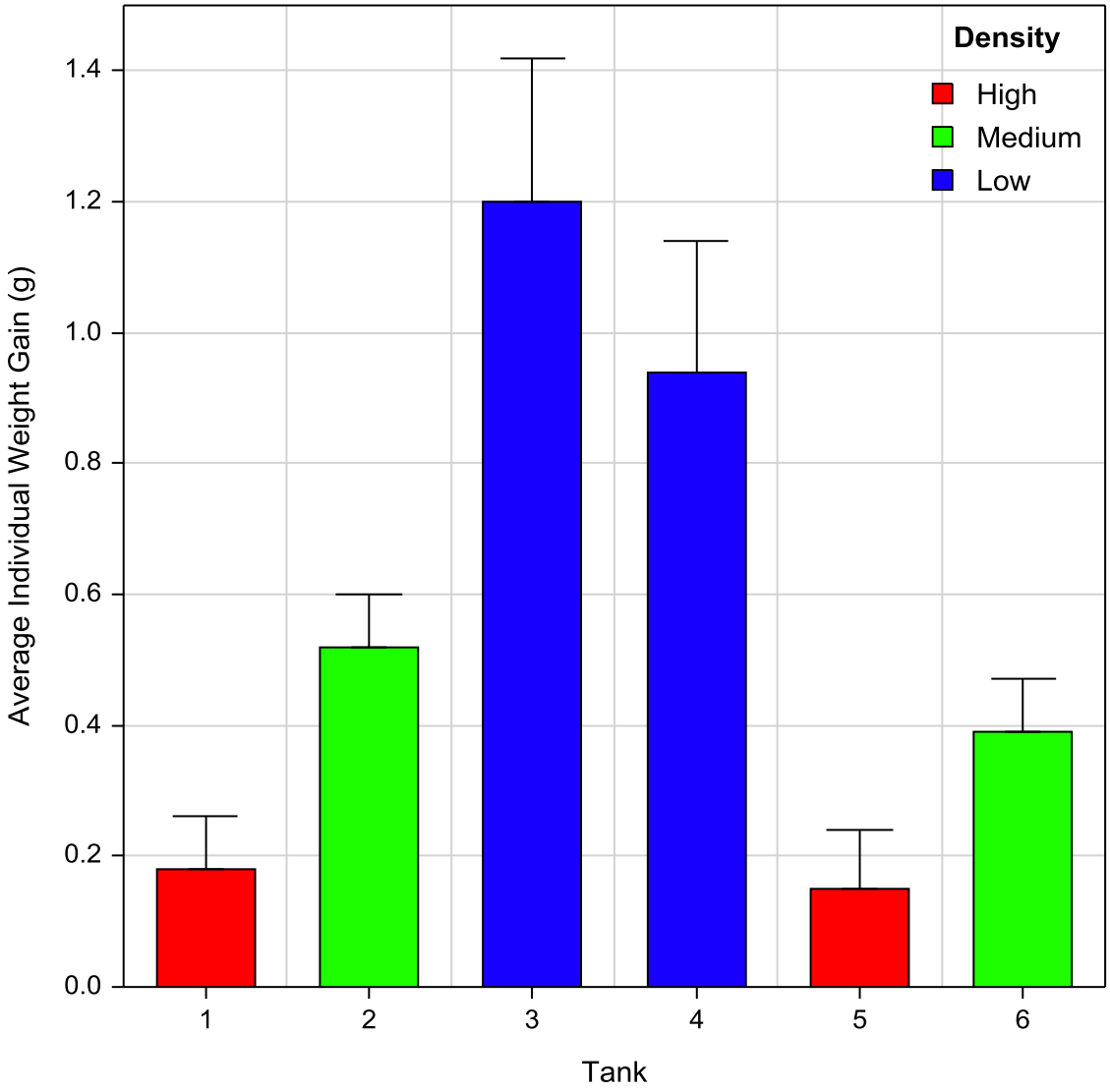

Figure 5. Average individual weight gain by tank.
Tanks 1 and 6 were high density, tanks 2 and 6 medium density, and tanks 3 and 4 low density. Error bars represent one standard error of the mean.

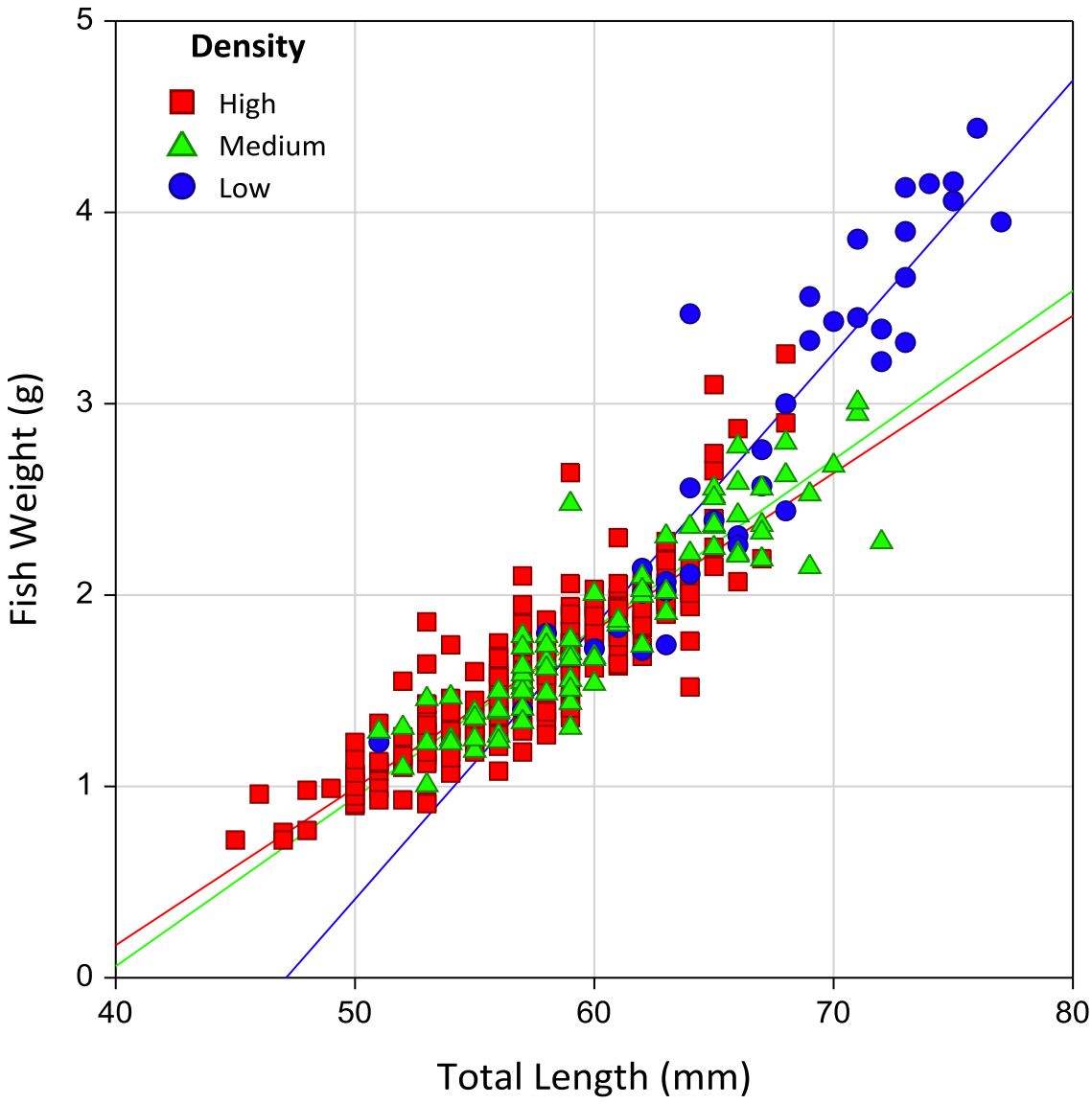

Figure 6. Observed Total length versus Fish Weight relationships for *Notropis topeka* by density. Regression parameter estimates appear in Table 7.

Table 7. Linear regression parameter estimates for the *Notropis topeka* total length versus weight relationship.
95% confidence intervals for the estimates are shown in parentheses. Model is Fish Weight in g = Intercept + Slope * Total Length in mm. All parameters were statistically significant ($p < 0.0001$).

Density	Intercept	Slope	R-squared
High	-3.1213 (-3.4700, -2.7726)	0.0822 (0.0762, 0.0883)	0.76
Medium	-3.4666 (-4.0081, -2.9252)	0.0883 (0.0793, 0.0972)	0.83
Low	-6.7209 (-8.0765, -5.3653)	0.1426 (0.1225, 0.1627)	0.86

eDNA Measurements

For each event, three of the five replicate samples from each tank were extracted and tested using the *N. topeka* qPCR endpoint assay for CytB. We quantitated total DNA concentrations of the extractions using a Qubit spectrophotometer and Qubit dsDNA HS Assay kits (Life Technologies, Inc.). This assay has a flourescent dye that binds to double stranded DNA molecules and increases in signal with concentration. Recovered total eDNA concentrations ranged from below the detection limit (< 0.5 ng/mL) to 825 ng/mL, and were generally higher during fish presence in the tanks, intermediate before stocking, and lowest after removal (Table 8). Total DNA concentrations from the field blanks and extraction control blanks were all below the detection limit of the Qubit assay. Extractions from tank 4 yielded the highest concentrations of total eDNA throughout the study. Total DNA concentrations of the extracts generally increased from the pre stocking condition through the first week (Figure 7). Concentrations of the extract total DNA 27 days after removal of the fish (day 375 after stocking) were generally lower than initial, pre-stocking concentrations. These concentrations represent the total amount of DNA recovered in extractions. *N. topeka* DNA is one potential component of the total DNA, with

other potential components including DNA fragments from numerous unicellular (e.g., algae) and multicellular organisms (e.g., macroinvertebrates) present in outdoor mesocosms.

Using eDNA production rate values recently measured in silver and bigheaded carp by Klymus et al (2015) as a starting point, sub-adult and juvenile fish produce between 10^4 and 10^8 DNA copies per hour, regardless of water temperature. Taking the median of the range, 10^6 copies / hr, and assuming a tank volume of 10 m^3 = 10^7 mL, an estimated production rate might be 0.1 copies / mL per hour for the bigheaded carp amplicon used in the Klymus et al. study. This amplicon has a molecular weight of 138,344.9 g/mol (calculated via Oligoanalyzer 3.1), suggesting a per fish production rate of:

$$0.1\frac{copies}{mL*hr}\ x\frac{mol}{6.022\ x\ 10^{23}\ copies}\ x\ 138{,}344.9\frac{g}{mol}\ x\ 10^9\frac{ng}{g}\ = 2.3\ x\ 10^{-11}\frac{ng}{mL*hr}$$

For the high, medium, and low density tanks (ostensibly 80, 40, and 20 fish), this translates to production rates of 18 x 10^{-10} ng /mL/hr, 9.2 x 10^{-10} ng /mL/hr, and 4.6 x 10^{-10} ng /mL/hr), respectively. These rates equate to a theoretical range of (432 to 110) x 10^{-10} ng /mL of Topeka shiner DNA per 24 hour period. Amounts of total DNA recovered from the tanks during fish presence ranged from 750 ng/mL to 75 ng/mL, suggesting that Topeka shiner DNA should make up less than one billionth of the recoverable DNA present in the water column by weight, even in the absence of losses from degradation and partitioning.

Using quantitative PCR, concentrations of *Notropis topeka* eDNA were calculated on a copies per mL basis, then converted to ng/mL using the molecular weight of the sequence amplified by the assay (25075.2 g/mol for the Ntop CytB 495/578 primer pair) (Table 9). Based on this conversion and the observed range of total eDNA concentrations (Table 8), *Notropis topeka* eDNA constituted less than 1 x 10^{-9} percent of the extracted DNA by weight per milliliter. Such small values are in line with both the theoretical calculation above and previous observations (Paul et al. 1987, Corinaldesi et al. 2005, Turner et al. 2014) that the vast majority of DNA extracted from environmental samples is likely associated with

microorganisms, rather than macro-organisms. Despite the small percentage by weight, macro-organismal DNA is still recoverable and amplifiable from water column samples in which the fish are present.

Table 8. Representative summary statistics for total extracted eDNA concentrations (ng/mL) for before, during, and after fish presence.

High, medium, and low density treatments are color coded red, green, and blue respectively. A dash indicates the concentration was below the detection limit (< 0.5 ng/mL) of the Qubit assay.

Fish Presence	Density	Tank	Mean	Standard Error	Count	Minimum	25th Percentile	Median	75th Percentile	Maximum
Before Fish Stocking	High	1	265	26.4	3	236	236	242	318	318
	High	5	350	36.8	3	282	282	361	408	408
	Medium	2	245	9.64	3	230	230	242	263	263
	Medium	6	91.8	54.2	3	30.5	30.5	45	200	200
	Low	3	155	12.3	3	132	132	159	174	174
	Low	4	277	60.0	3	184	184	257	389	389
Fish Present	High	1	280	42.4	12	58.5	122	307	387	483
	High	5	336	64.5	12	34.0	127	338	530	665
	Medium	2	375	51.4	12	-	285	407	510	625
	Medium	6	286	40.3	12	34.5	212	282	399	535
	Low	3	270	32.6	12	-	236	249	348	466
	Low	4	579	46.4	12	398	417	613	690	825
After Fish Removal	High	1	8.83	8.83	3	-	-	26.5	26.5	26.5
	High	5	148	38.2	3	72.0	72.0	180	192	192
	Medium	2	47.5	13.8	3	29.0	29.0	39	74.5	74.5
	Medium	6	77.0	26.1	3	32.5	32.5	75.5	123	123
	Low	3	175	30.0	3	127	127	167	230	230
	Low	4	376	169	3	204	204	210	715	715

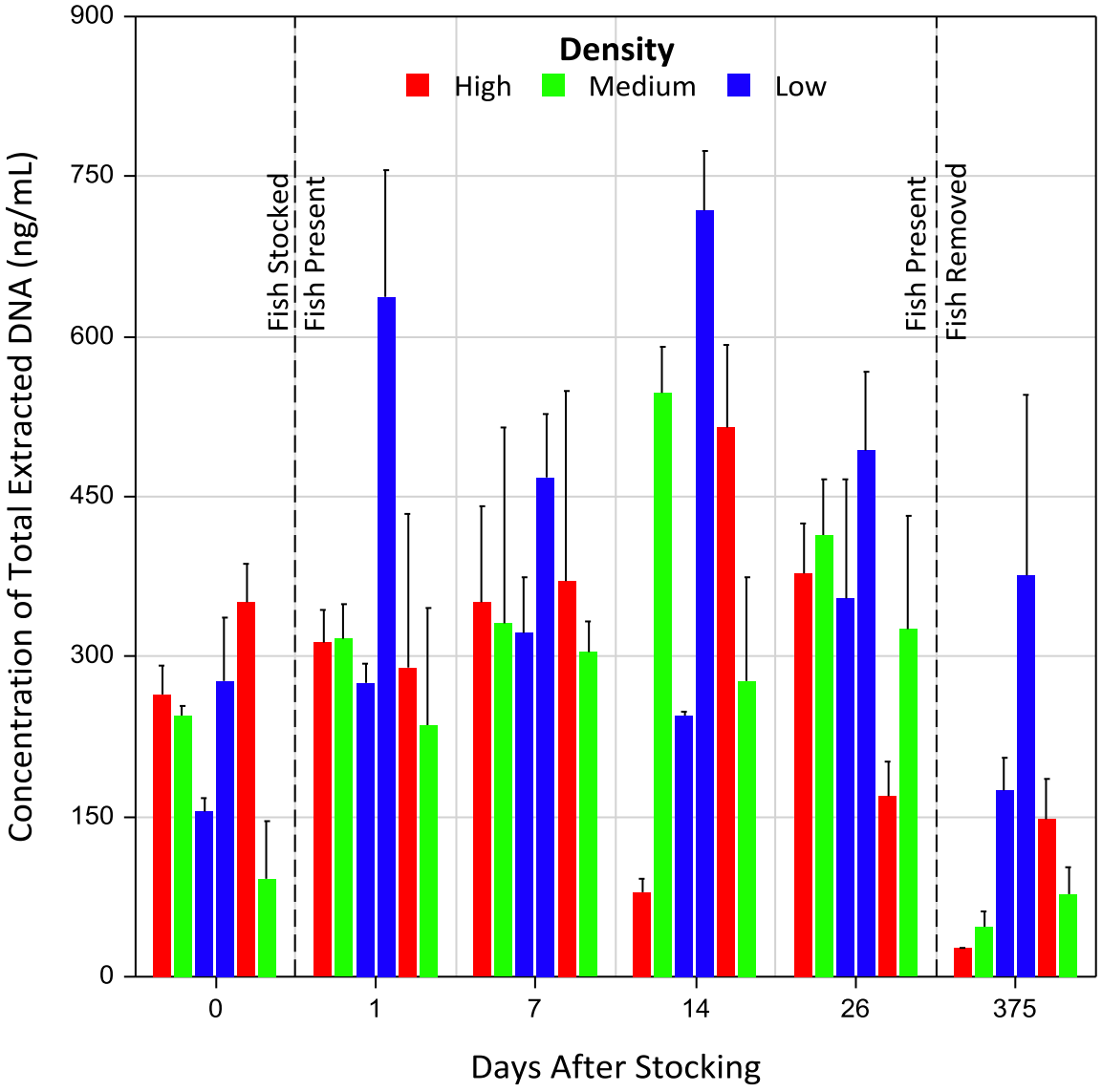

Figure 7. Concentration of total extracted DNA (ng/mL) by tank over time.
For each sampling event, bars represent the mean concentration of extracted total DNA, arrayed by tank from left to right (i.e., 1, 2, ..., 6). Error bars represent one standard error of the mean. Colors represent density treatment - red is high density (tanks 1 and 5), green is medium density (tanks 2 and 6), and blue is low density (tanks 3 and 4). Dashed lines indicate the presence of *N. topeka* in the experimental tanks.

Table 9. Calculated eDNA concentrations (ng/mL) for *Notropis topeka* during fish presence. High, medium, and low density treatments are color coded red, green, and blue respectively. Values calculated by converting from copies per reaction using the molecular weight of the *Notropis topeka* CytB 495/578 amplicon used in the assay (25,075.2 g/mol).

Days After Stocking	Density	Count	Mean	Standard Error	Maximum
1	hi	17	4.11E-08	5.35E-09	7.38E-08
	med	16	1.87E-08	3.45E-09	4.26E-08
	low	16	1.44E-08	3.33E-09	5.61E-08
3	hi	11	4.74E-09	1.02E-09	1.20E-08
	med	6	3.40E-09	1.23E-09	7.33E-09
	low	9	3.50E-09	6.60E-10	6.36E-09
6	hi	8	1.73E-09	2.98E-10	3.05E-09
	med	4	1.62E-09	5.63E-10	2.64E-09
	low	5	2.42E-09	4.60E-10	4.06E-09
7	hi	7	1.07E-09	3.05E-10	2.48E-09
	med	3	1.62E-09	1.55E-09	4.72E-09
	low	2	1.19E-09	9.58E-10	2.15E-09
8	hi	4	5.39E-09	2.39E-09	9.78E-09
	med	4	3.47E-09	3.46E-09	1.38E-08
	low	1	5.61E-09		5.61E-09
14	hi	14	7.51E-09	1.09E-09	1.58E-08
	med	5	5.12E-09	1.93E-09	1.11E-08
	low	8	7.33E-09	1.26E-09	1.47E-08
26	hi	16	6.84E-09	1.35E-09	2.21E-08
	med	6	6.14E-09	1.44E-09	1.11E-08
	low	6	5.45E-09	1.33E-09	8.99E-09
335	hi	5	1.18E-08	7.58E-09	3.84E-08
	med	1	3.33E-11		3.33E-11
	low	3	2.60E-09	2.36E-09	7.31E-09

Density groups were based on initial stocking rate, hi = 80 fish, med = 40 fish, low = 20 fish.

Endpoint Assay

Detection/Nondetection was determined by comparison of endpoint assay melt curve results with predicted amplicon melt curve peaks and melt peaks of positive controls. Assays were judged to indicate presence even if suppressed amplification (i.e., increased cycle times) indicated relatively low quantities of target DNA.

Results from the endpoint assay for CytB showed several trends (Table 10, Figure 8). First, none of the tanks tested positive for the presence of *N. topeka* prior to stocking of fish, confirming that the baseline no-fish condition tested as an absence according to the assay (data not shown). Second, an initial spike of positive hits was observed on the first day after stocking. This result is consistent with a pulse of genetic material released by the fish under the stress of handling and transfer, and such an initial spike has been shown in similar eDNA studies of other fish species (Takahara et al. 2012, Thomsen et al. 2012). Following the initial spike, there was a drop off of the number of positives after one week, which is also consistent with the attenuation of the first spike signal as the initial DNA pulse is diluted and degraded in the tank. As the fish remained in place, the number of positives again increased for the high density tanks, and remained at a lower, but still detectable levels for the lower density treatments. Tanks with higher stocking densities of fish (both by count and by biomass) showed more positive hits in the endpoint assay for CytB at 7, 14, and 26 days (Table 10, Figure 8). This result is consistent with a build-up of *N. topeka* eDNA at higher densities. After 26 days, *N. topeka* eDNA was detectable in four of the six tanks and in all density treatments. In the absence of a net accumulation in lower density tanks, it is hypothesized that eDNA production may have been sufficient for detection, but not sufficient to overcome in-tank degradation rates and provide a surplus of material.

Table 10. Endpoint assay detection/nondetection data for *Notropis topeka* by tank.
A positive result (shown as +) indicates detection of *N. topeka* CytB DNA. Density treatments were "High" (80 fish) for tanks 1 and 5, "Medium" (40 fish) for tanks 2 and 6, and "Low" (20 fish) for tanks 3 and 4.

Tank	Density Treatment	Pre-Stocking	Days Post Stocking			
		0	1	7	14	26
1	High		+ + +	+ +	+ + +	+ + +
5			+ + +	+	+ + +	+ +
2	Medium		+ + +		+	+
6			+	+		
3	Low		+ + +			
4			+ + +	+ +	+	+ +

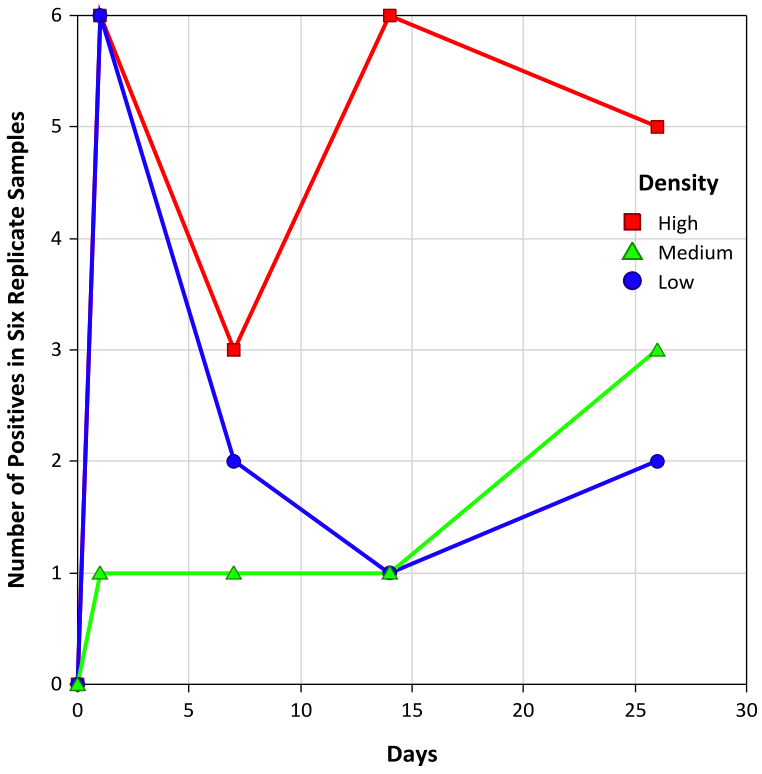

Figure 8. Endpoint asssay detection/nondetection data for *Notropis topeka* by density treatment.
Values are number of positive results in the CytB endpoint assay. Six replicate samples (three in each of two tanks) were extracted and tested for each density treatment at each sampling event. Positive results indicate presence of *N. topeka* CytB DNA, with numbers closer to six indicating a stronger signal of *N. topeka* presence.

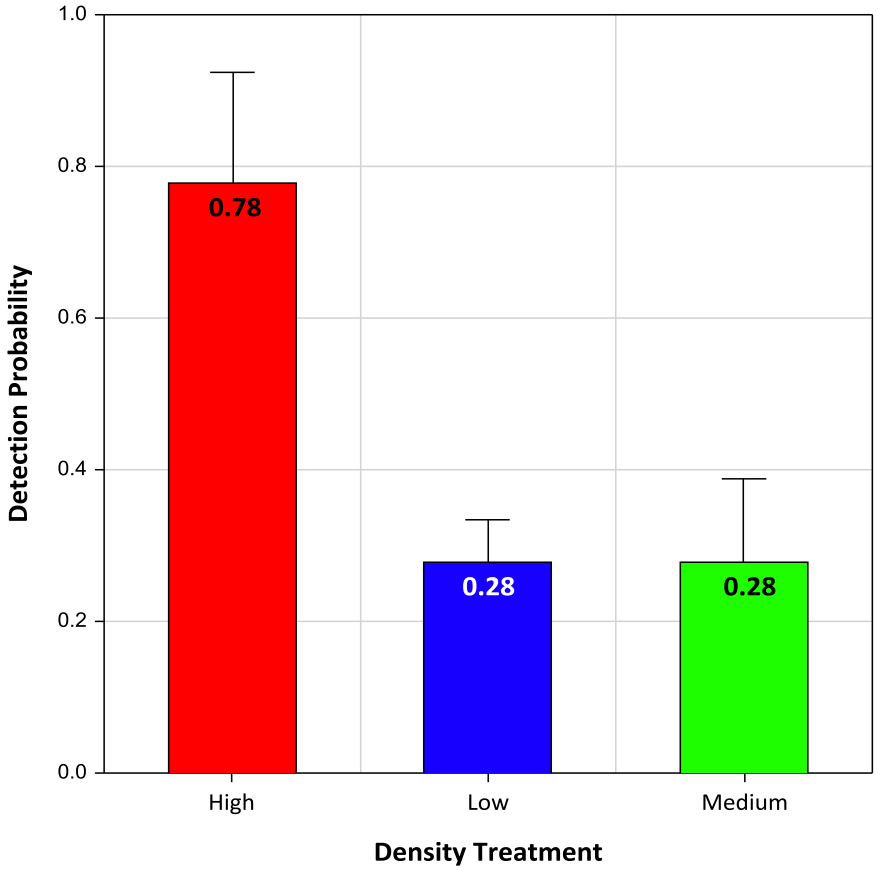

Figure 9. Detection probability of CytB qPCR endpoint assay based on 18 observations for each density treatment (6 samples per treatment x 3 sampling events).
Mean detection probabilities were calculated using detect/nondetect data for the 7 day, 14 day, and 26 day sampling events to remove effects of both the prestocking and initial eDNA spike conditions. Mean values are shown with error bars representing one standard error of the mean.

After removal of prestocking nondetects and initial eDNA spike detection results, the mean probability of detection (*p*) in the remaining three sampling events was significantly higher in the high density treatment tanks (0.78 ± 0.15) as compared to the medium and low density tanks (0.28 ± 0.11 and 0.28 ± 0.06, respectively) (Table 10). The mean nondetection probability (1 - *p*) for each treatment then was 0.22, 0.72, and 0.72 for the high, medium, and low treatments, respectively. Increased probability of detection may be associated with the increased proximity of live fish to the sampling apparatus during sample collection, with increased suspension of *N. topeka* eDNA in the water column, with increased eDNA production due to altered behaviors or larger schools at high densities, or other factors. Since

collection, extraction, and amplification methods were the same across treatments and sampling events, and since all of the positive and negative controls amplified as expected, the difference in detection probability of the endpoint assay with density appears to be a real phenomenon.

Degradation of eDNA was also examined using the endpoint assay. After 335 days to develop a long-term occupancy load of *N. topeka* eDNA, the fish were removed by seining. eDNA samples were again taken 27 days after removal, then extracted and tested using the qPCR endpoint assay for CytB. Only 3 of the 18 samples (i.e., three replicates for each of six tanks) tested positive -- one each in tank 5, tank 2, and tank 4. These results show that *N. topeka* eDNA is recoverable and detectable at least 27 days after removal of fish under field conditions, regardless of the density at which those fish were present and even after long periods of site occupancy. As before, positive controls were positive and negative controls were negative in this assay.

Quantitative PCR Assay

Water column eDNA as measured by the quantitative PCR assay followed similar trends to the endpoint assay for detection/nondetection whether examined using all qPCR replicates (Table 11, Figure 10) or by grab samples alone (Table 12, Figure 11), as did quantitative estimates of eDNA concentrations (Table 13, Figure 12). An initial spike immediately following stocking was observed, followed by a rapid decrease in concentration with a subsequent rapid increase followed by consecutively similar *N. topeka* eDNA levels. Of 24 total sampling events (8 time periods x 3 densities), 19 had positive detections in at least one grab sample (Table 11), yielding an overall probability of detection (p) of 0.79, with a corresponding nondetection probability (1 - p) of 0.21. On days 14 and 26 after the initial spike had abated and the concentration of *N. topeka* eDNA had built up in the tanks, 14 of all 18 grab samples (78%) and at least 1 grab sample in every tank had a positive detection. However, at day 335, only 1 grab sample from all 6 tanks tested positive. Since all tanks exhibited an increase in fish size (Table 6, Figure 5) with a presumably concurrent increase in eDNA production rate as shown by Klymus et al. (2015), this

decline in detection is hypothesized to be due to increased loss rates at day 335 compared to day 26. Increased loss rates may be attributed to (1) increased biological degradation from higher enzyme concentrations associated with microbial growth during the 300+ days, (2) increased partitioning due to increased surface area of biofilms and organic particles associated with microbial growth and other detritus, and (3) increased physical degradation from higher temperatures and increased solar radiation.

Calculated concentrations of *N. topeka* eDNA declined from initial highs of 536 +/- 91.9 copies/mL and 102 +/- 27.3 copies/mL in the highest and lowest tanks (1 and 3, respectively) to around the detection limit by day 6, then remained there through day 8 (Table 13, Figure 12). Between day 8 and day 14, all but 1 tank increased from the detection limit and remained there at day 26. *N. topeka* eDNA concentrations did not reach initial spike levels at any time subsequent to day 1. Relative standard errors (i.e. standard error / mean) were relatively high (17% to 30%) for most sampling events (Table 13). For sampling events with a majority of nondetections (e.g., days 6 and 7), relative standard errors were lower (2% to 24%), as would be expected. Relative standard errors at day 335 were considerably higher than the rest of the experiment (20% to 80%), which may be due to increased patchiness of material caused by adherence of ejecta or other eDNA bearing material to detritus that had built up over the course of the experiment. The 95% method detection limit (MDL) for the Ntop CytB assay used in this study is 27 copies per reaction, which is comparable to the 95% MDL (30 copies/rxn) of a published assay for other fish extracted by similar methods from KUFS waters (Turner et al. 2015).

Table 11. Numbers of positive detections in all replicates by density treatment over time.
Numbers in parentheses indicate the number of PCR replicates for each *Notropis topeka* CytB 495/578 trial.

Density Treatment	Days After Stocking								Totals
	1	3	6	7	8	14	26	335	
High	17 (18)	6 (18)	1 (18)	0 (18)	2 (18)	13 (18)	13 (18)	1 (9)	53 (135)
Medium	13 (18)	2 (18)	0 (18)	1 (18)	1 (18)	3 (18)	5 (18)	0 (6)	25 (132)
Low	15 (18)	5 (18)	1 (18)	0 (18)	1 (18)	8 (18)	5 (18)	0 (9)	35 (135)
Medium And Low Combined	28 (36)	7 (36)	1 (36)	1 (36)	2 (36)	11 (36)	10 (36)	0 (15)	60 (267)
Totals	45 (54)	13 (54)	2 (54)	1 (54)	4 (54)	24 (54)	23 (54)	1 (24)	113 (402)

Density treatment refers to the initial stocking rate of the tanks, high = 80 fish, medium = 40 fish, and low = 20 fish.
Medium and Low Combined is the sum of medium and low.

Table 12. Numbers of grab samples with positive detections by density treatment over time.
Numbers in parentheses indicate the number of grab samples taken for each *Notropis topeka* CytB 495/578 trial. A grab sample was deemed positive if at least one of its three PCR replicates was positive.

Density Treatment	Days After Stocking								Totals
	1	3	6	7	8	14	26	335	
High	6 (6)	4 (6)	1 (6)	0 (6)	2 (6)	6 (6)	6 (6)	1 (3)	26 (45)
Medium	6 (6)	1 (6)	0 (6)	1 (6)	1 (6)	3 (6)	3 (6)	0 (2)	15 (44)
Low	6 (6)	3 (6)	1 (6)	0 (6)	1 (6)	5 (6)	5 (6)	0 (3)	21 (45)
Medium And Low Combined	28 (12)	7 (12)	1 (12)	1 (12)	2 (12)	11 (12)	10 (12)	0 (5)	36 (89)
Totals	18 (18)	8 (18)	2 (18)	1 (18)	4 (18)	14 (18)	14 (18)	1 (8)	62 (134)

Density treatment refers to the initial stocking rate of the tanks, high = 80 fish, medium = 40 fish, and low = 20 fish.
Medium and Low Combined is the sum of medium and low.

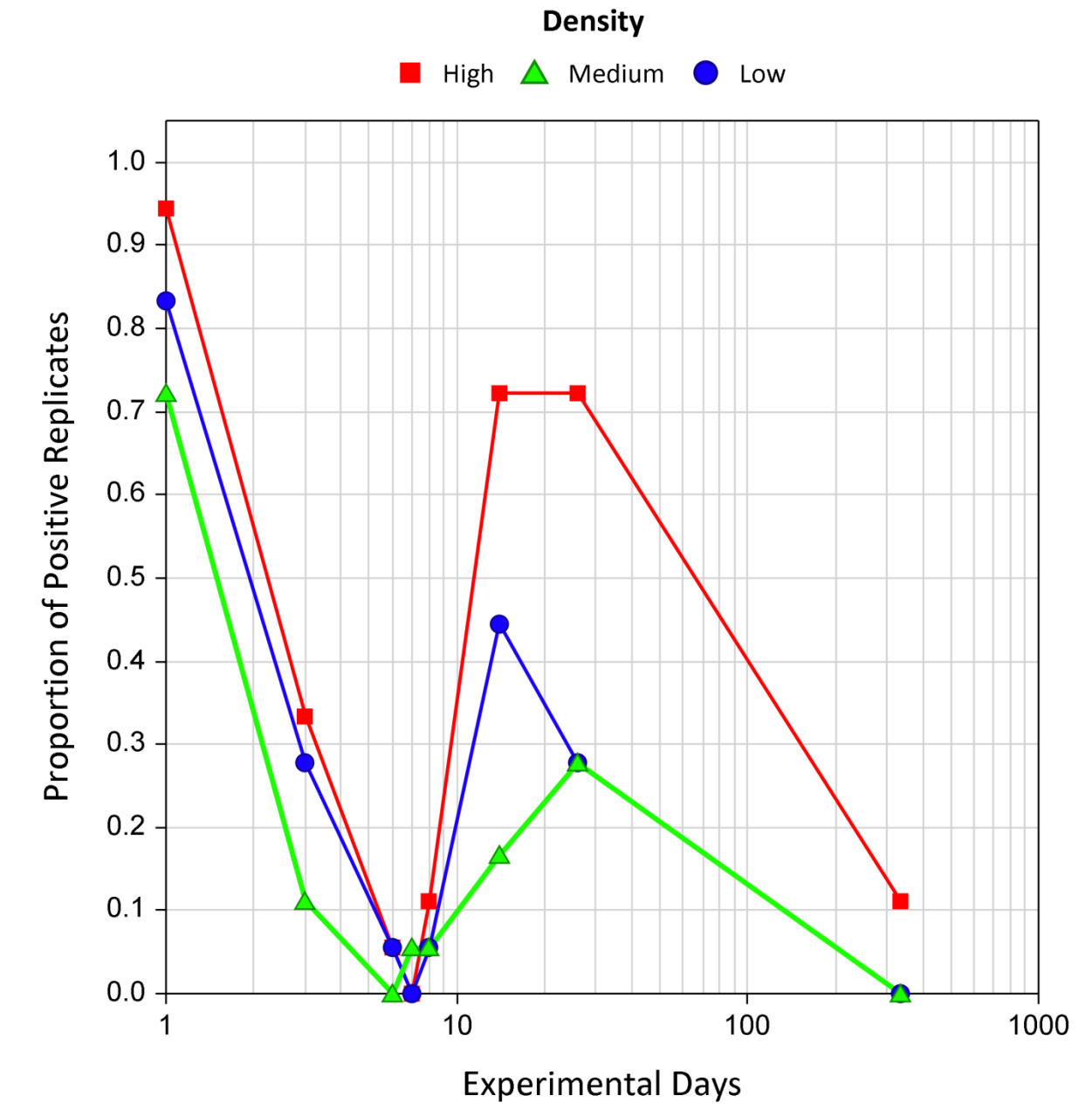

Figure 10. Proportion of all PCR replicates with positive detections by density treatment.
Each grab sample had three PCR replicates, yielding 18 replicates for the high density treatment (6 grabs x 3 replicates/grab) and 36 replicates for the medium and low density treatments combined (6 grabs from medium density and 6 grabs from low density x 3 replicates/grab).

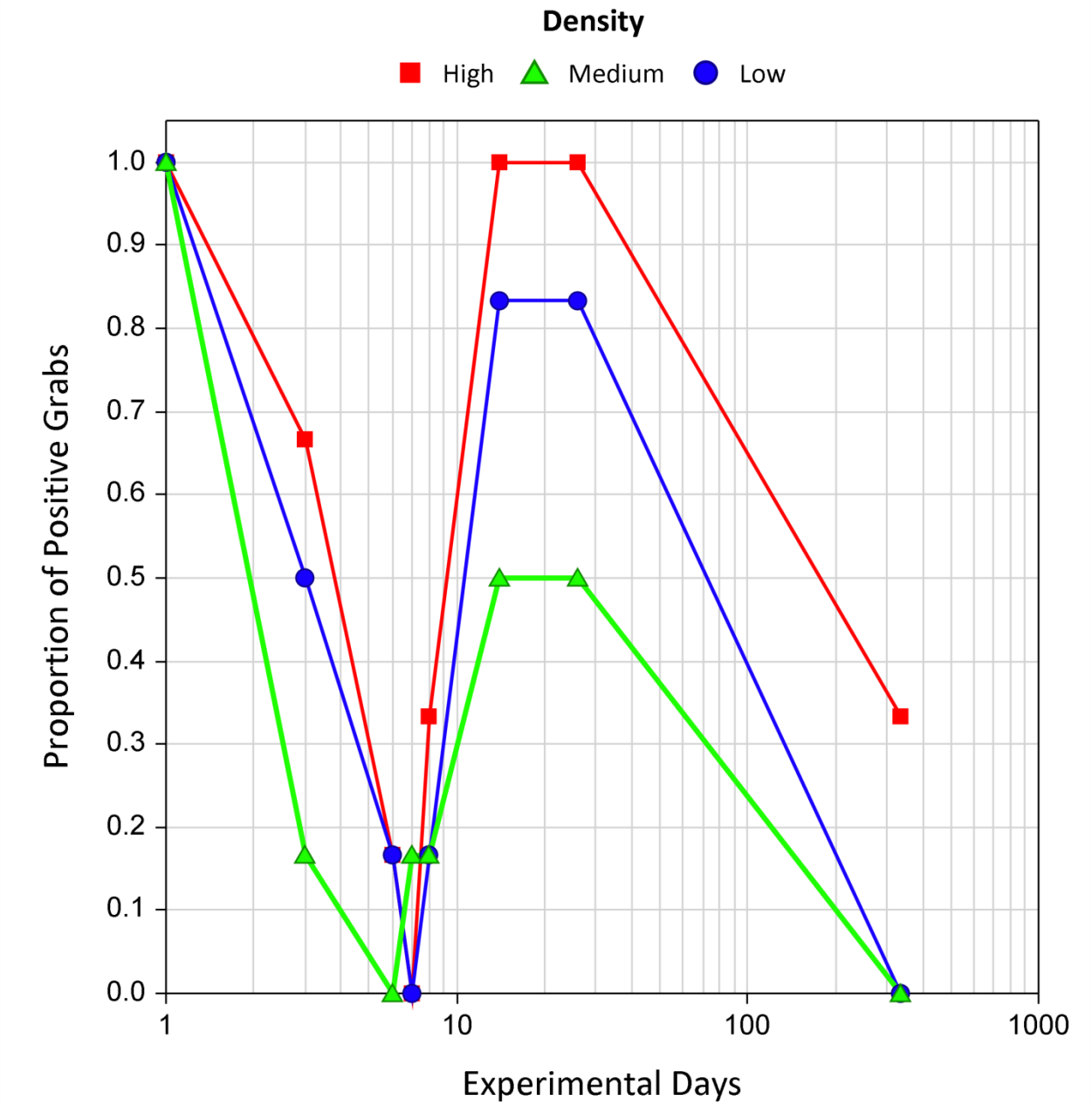

Figure 11. Proportion of detections by density treatment.
Proportions represent the number of grab samples with a positive detection for *Notropis topeka* DNA out of 6 grabs for the high density condition (3 grabs/tank x 2 tanks) and 12 grabs for the medium/low density condition (3 grabs/tank x 2 medium density tanks and 2 low density tanks). A grab sample was deemed positive if at least one of its PCR replicates was positive.

Table 13. Calculated statistics for *Notropis topeka* eDNA concentrations (copies / mL) in the water column by tank over time.
Count indicates the number of PCR replicates used for calculation, SD indicates standard deviation, SE indicates standard error of the mean, and RSE indicates relative standard error (SE / mean). For purposes of calculation, non-detects were replaced with half the detection limit.

Days After Stocking	Tank	Density Treatment	Count	Mean	Median	SD	SE	RSE
1	1	High	9	536	673	276	91.9	0.171
1	2	Medium	9	167	130	145	48.4	0.291
1	3	Low	9	102	106	82	27.3	0.268
1	4	Low	9	210	130	201	66.9	0.319
1	5	High	9	398	417	284	94.6	0.238
1	6	Medium	9	236	296	192	64.2	0.272
3	1	High	9	24.7	18.0	14.1	4.71	0.191
3	2	Medium	9	15.4	18.0	5.53	1.84	0.120
3	3	Low	9	24.9	18.0	19.3	6.44	0.259
3	4	Low	9	35.1	30.3	21.4	7.14	0.203
3	5	High	9	58.9	43.3	45.0	15.0	0.255
3	6	Medium	9	35.9	18.0	28.5	9.48	0.264
6	1	High	9	17.2	18.0	4.07	1.36	0.0786
6	2	Medium	9	18.5	18.0	1.38	0.460	0.0249
6	3	Low	9	19.1	18.0	3.15	1.05	0.0552
6	4	Low	9	23.1	18.0	10.61	3.54	0.153
6	5	High	9	21.2	18.0	8.29	2.76	0.130
6	6	Medium	9	18.2	18.0	8.22	2.74	0.151
7	1	High	9	16.3	18.0	7.62	2.54	0.156
7	2	Medium	9	16.0	18.0	5.97	1.99	0.124
7	3	Low	9	18.0	18.0	0	0	0
7	4	Low	9	17.2	18.0	5.98	1.99	0.116
7	5	High	9	15.7	18.0	5.09	1.70	0.108
7	6	Medium	9	20.5	18.0	14.6	4.87	0.238

Days After Stocking	Tank	Density Treatment	Count	Mean	Median	SD	SE	RSE
8	1	High	9	18.0	18.0	0	0	0
8	2	Medium	9	12.0	18.0	8.97	2.99	0.249
8	3	Low	9	18.0	18.0	0	0	0
8	4	Low	9	23.5	18.0	16.5	5.487	0.234
8	5	High	9	38.8	18.0	43.0	14.3	0.369
8	6	Medium	9	34.5	18.0	49.4	16.5	0.478
14	1	High	9	80.9	80.8	55.2	18.4	0.228
14	2	Medium	9	17.2	18.0	3.7	1.2	0.0714
14	3	Low	9	58.9	45.7	54.0	18.0	0.305
14	4	Low	9	39.3	18.0	34.4	11.5	0.292
14	5	High	9	67.5	52.7	52.7	17.6	0.261
14	6	Medium	9	43.0	18.0	42.5	14.2	0.330
26	1	High	9	99.9	66.6	74.3	24.8	0.248
26	2	Medium	9	21.9	18.0	13.7	4.55	0.208
26	3	Low	9	29.5	18.0	30.7	10.2	0.347
26	4	Low	9	38.1	18.0	33.1	11.0	0.289
26	5	High	9	50.1	31.1	42.9	14.3	0.285
26	6	Medium	9	51.3	18.0	44.7	14.9	0.290
335	1	High	6	53.5	18.0	86.9	35.5	0.664
335	2	Medium	6	18.0	18.0	0	0	0
335	3	Low	6	24.6	18.0	31.9	13.0	0.5
335	4	Low	9	18.0	18.0	0	0	0
335	5	High	9	63.1	18.0	149	49.78014	0.789
335	6	Medium	6	15.1	18.0	7.19	2.93	0.195

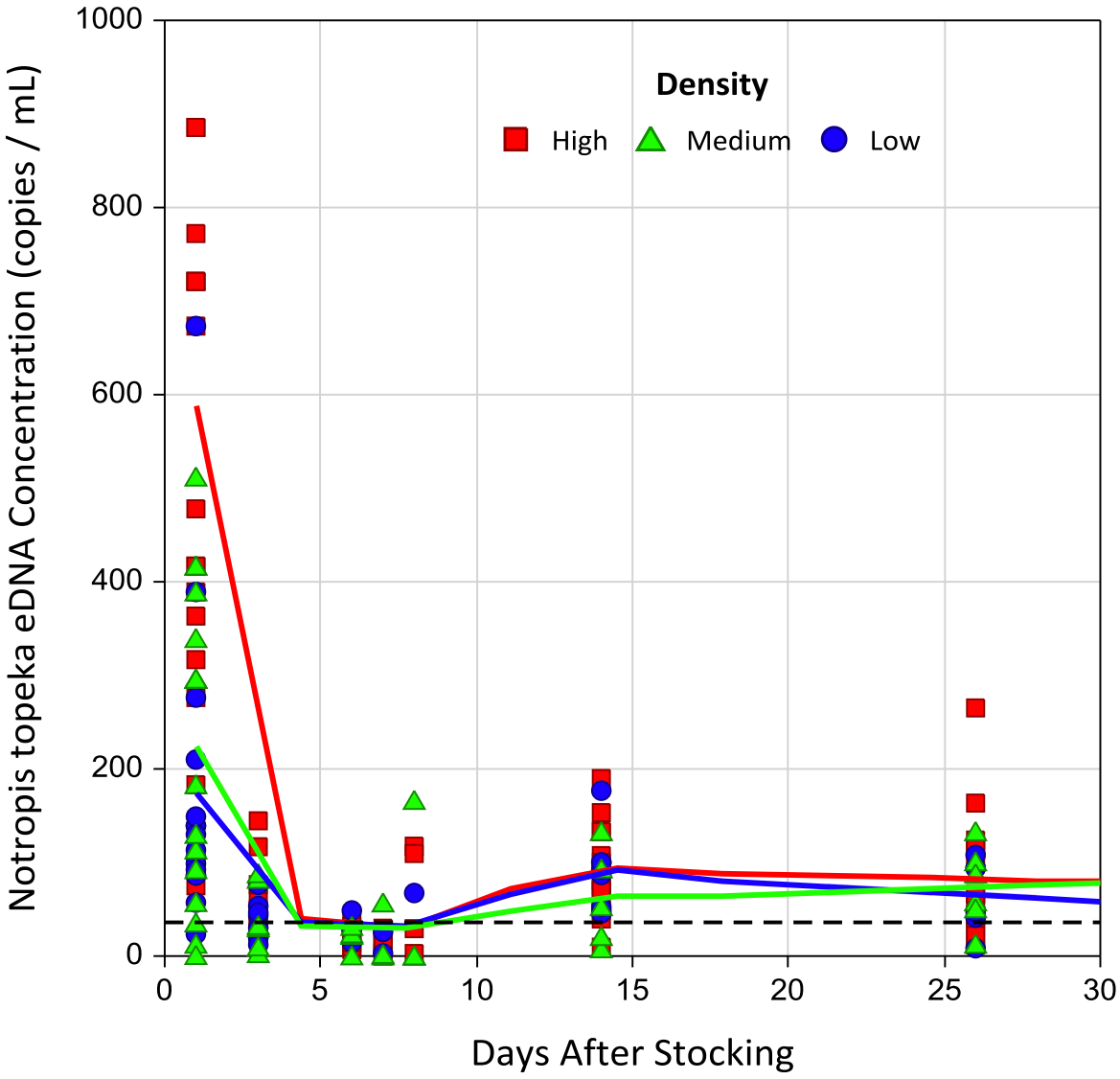

Figure 12. Concentration of *Notropis topeka* eDNA by density treatment over time.
Red squares, green triangles, and blue circles represent high, medium, and low density treatments, respectively. Solid lines are LOESS smoothed curves to show the general trends of the data, with colors to match their respective density treatments. The dashed line represents the method detection limit (MDL) of 36 copies/mL. Non-detects were replaced with half detection limits for analytical purposes.

Modeling of Detection Probability

The probability of detection was modeled as a binomial variable (i.e., detection vs. non-detection) using generalized linear mixed modeling with the logit link function with the lme4 package in R (R Core Team 2015). Based on the Akaike coefficient and comparison of nested models, the best fit model included days since stocking, number of fish stocked, fish biomass stocked, and tank as fixed effects and water temperature as a random effect. The model shows significant effects of water temperature ($p = 0.0027$), the number of days since stocking ($p = 0.0013$), the number of fish stocked ($p = 0.021$), the biomass of fish stocked ($p = 0.019$), and a tank effect ($p = 0.007$) (Table 15). Despite high correlation Tank, number of fish, and fish biomass (Table 16), removal of any of the terms significantly reduced the deviance predicted by the model ($p < 0.01$) (based on ANOVA of nested models). Therefore, tank specific effects as well as both the number and biomass of fish appear to be important predictors for detection probability of *N. topeka* in this study.

Table 14. Generalized linear mixed models of detection for days 6 to 26.
Water temperature is modeled as a covariate. Plus signs indicate additional model terms, and colons denote interaction terms. Lower Akaike Information Coefficient (AIC) indicates nominally better fit to be confirmed by comparison of nested models. Selected model shown in bold.

Model	AIC	Model Specification
1	131.1	Detection ~ (1 | Water Temperature) + Grab + Replicate + Days + Count Stocked + Biomass Stocked + Average Weight + Tank
2	138.7	Detection ~ (1 | Water Temperature) + Grab + Replicate + Days + Count Stocked + Biomass Stocked + Average Weight + density
3	131.1	Detection ~ (1 | Water Temperature) + Grab + Replicate + Days + Count Stocked + Biomass Stocked + Average Weight + Tank
4	133.3	Detection ~ (1 | Water Temperature) + Days + Count Stocked + Biomass Stocked + Average Weight + density
5	126.6	Detection ~ (1 | Water Temperature) + Days + Count Stocked + Biomass Stocked + Average Weight + Tank
6	**125.1**	**Detection ~ (1 | Water Temperature) + Days + Count Stocked + Biomass Stocked + Tank**
7	127	Detection ~ (1 | Water Temperature) + Days + Count Stocked + Biomass Stocked + Tank + Tank:Biomass Stocked
8	138	Detection ~ (1 | Water Temperature) + Days + Count Stocked + Biomass Stocked + density
9	130.1	Detection ~ (1 | Water Temperature) + Days + Count Stocked + Tank
10	129.8	Detection ~ (1 | Water Temperature) + Days + Biomass Stocked + Tank
11	128.5	Detection ~ (1 | Water Temperature) + Days + Tank
12	130.1	Detection ~ (1 | Water Temperature) + Days + Count Stocked + Tank
13	129.8	Detection ~ (1 | Water Temperature) + Days + Biomass Stocked + Tank
14	137.4	Detection ~ (1 | Water Temperature) + Days + Count Stocked + Biomass Stocked

Detection is detection probability, (i.e., proportion of detections)
Water Temperature is the in situ temperature of the tank.
Grab is the grab number for the sample, (i.e., first grab, second grab, or third grab)
Replicate is number of the qPCR replicate for a given sample (i.e, first well, second well, etc.)
Days is the number of days since stocking.
Count Stocked is the initial stocking number of *N. topeka* for each tank
Biomass Stocked is the initial stocking biomass of *N. topeka* for each tank
Tank is the number designation of the experimental tank (1, 2, 3, 4, 5, 6)
Density is the number of fish per tank

Table 15. Model fit information for the selected generalized linear mixed model for detection. Note that all terms remaining in the model are statistically significant (i.e., probability of values as extreme or more extreme than the observations are all less than 0.025). Z-value is the normalized z-score of the parameter estimate.

Parameter	Estimate	Standard Error	z value	p - value
Water Temperature	-7.46224	2.48256	-3.006	0.00265
Days	0.18835	0.05876	3.205	0.00135
Count Stocked	-0.35432	0.15336	-2.31	0.02087
Biomass Stocked	0.25852	0.11058	2.338	0.01939
Tank	0.96129	0.35706	2.692	0.0071

Table 16. Correlation matrix for coefficients in best-fit model for detection.

	Water Temp	Days	Count Stocked	Biomass Stocked
Days	-0.598			
Count Stocked	0.691	-0.248		
Biomass Stocked	-0.722	0.257	-0.997	
Tank	-0.898	0.416	-0.641	0.656

Theoretical Modeling of Water Column Concentration

Using a mass balance of the concentration of eDNA in the water column, the net concentraion is the sum of the background concentration and gains, less the losses:

$$Water\ Column\ eDNA = Background + Gain - Loss$$

More specifically,

$$Water\ Column\ eDNA = Background + (Inflow + Production + Resuspension) - (Partitioning + Decay + Outflow)$$

In the KUFS mesocosms, the tanks were filled initially then left to sit, so inflow and outflow are very small, and resuspension from wind, animal movements, and other generated currents is relatively minimal. Therefore,

$$\begin{aligned} Water\ Column\ eDNA &= (Production) - (Partitioning + Decay) \\ &= GAIN - LOSS \\ &= Logistic\ Growth - Exponential\ Decay \end{aligned}$$

Since partitioning and decay have been shown to be exponential (Matsui et al. 2001, Nielsen et al. 2007, Zhang et al. 2009, Strickler et al. 2015), and since production is based on ecological process that typically have an upper bound (e.g., cells with specific growth rates), the following theoretical model is proposed for eDNA concentration:

$$y(t) = \frac{A}{1+Be^{-Ct}} + D\ e^{-Ft}$$

where t is time, y(t) is the water column concentration of eDNA, and where A,B,C,D,F > 0. A and B are coefficients of growth, C is the rate of growth, D is a coefficient of loss, and F is the rate of loss.

Using the initial stocking condition and stabilization observed in the first 26 days of the experiment as an analog for a change in water column eDNA concentration (Figure 12), we see an exponential loss followed by a logistic gain (Figure 13). This pattern is consistent with the hypothesis that water column eDNA concentration is a net balance of loss and gain, with an upper bound to the gain imposed by some controlling factors like physical limitations (e.g., sinking of material, partitioning into other environmental compartments, physical saturation of the water column, direct cleavage of DNA by ultraviolet light, sorption to clays or other hydrolysis-mediating materials) and biological limitations (e.g., rapid hydrolysis by background concentrations of enzymes, direct ingestion of DNA bearing particles). If the observed water column concentration is in fact governed losses and gains, and if these losses and gains are dependent on differential rates, which are governed by environmental conditions with upper bounds on production, then some observable patterns should occur (Figure 14). For example, if inflow and outflow contributions are balanced, there should be a higher upper bound in low degradation conditions (e.g., winter) as compared to high degradation conditions (e.g., summer). Similarly, there should be periods of more rapid and less rapid change associated with transitional changes between these two conditions. Reduced water column concentrations of *N. topeka* eDNA observed in this study at day 335 with respect to day 26, despite long-term occupancy of the fish, are consistent with the hypothesis that there may be seasonal differences in the upper bound due to environmental conditions. It is also possible that the upper bound may be largely mediated by hydrolytic enzyme concentration, since enzymes have been shown to degrade DNA in the water column regardless of the presence of cells that originally produced them (Matsui et al. 2001). It would also be expected that there could be some extreme gain or loss events associated with ephemeral phenomena based on biological (e.g., seasonal spawning, fish kills) or physical factors (e.g., repartitioning from different environmental compartments by resuspension of material from sediments, pH-mediated changes in sorption, rapid changes in ultraviolet light penetration, rapid changes in temperature). Since these phenomena occur under naturally variable environmental conditions, diurnal and other small-scale fluctuations in both gain and loss rates would be expected, potentially making overall trends more difficult to discern.

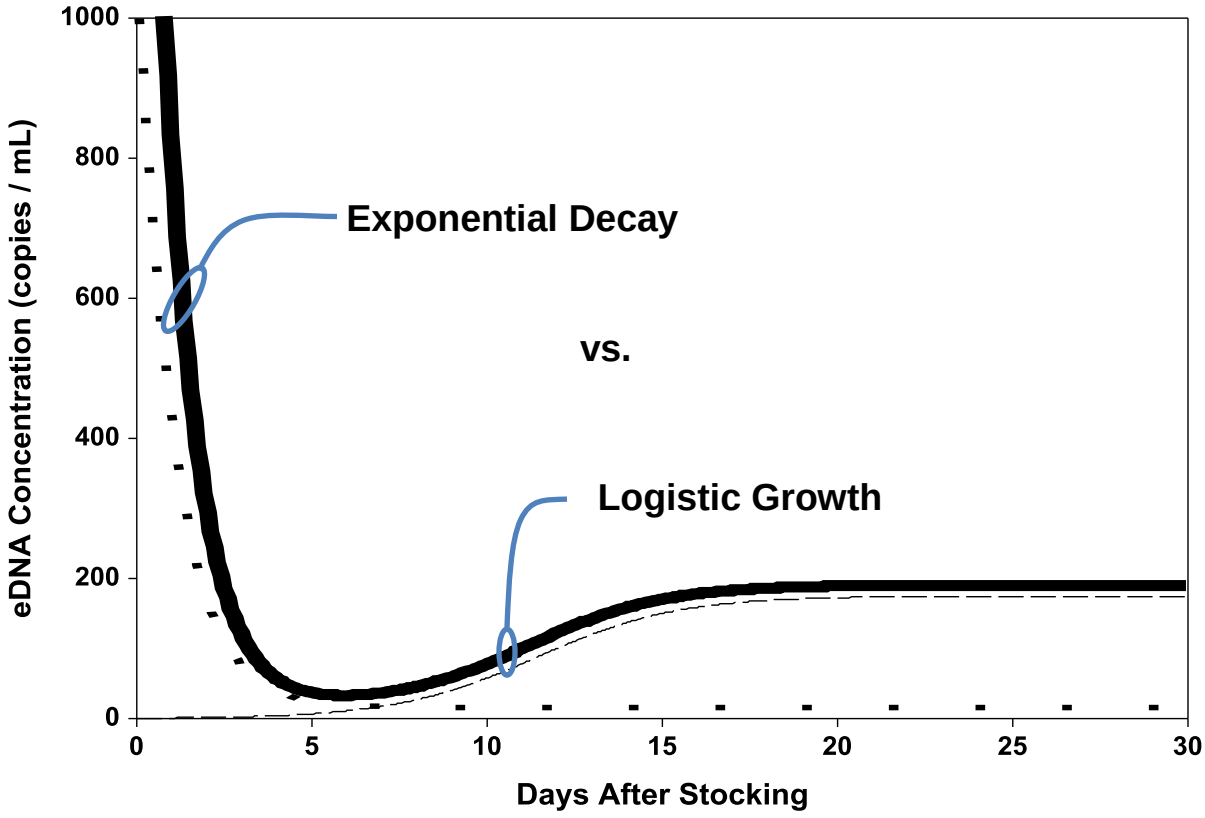

Figure 13. Observed response curve to initial stocking and theoretical response to other high production events.
The dotted line represents exponential losses due to physical and biological factors (e.g., sinking, sorption, photodegradation, hydrolysis by enzymes), the dashed line represents logistic increase in water column eDNA concentration by production (e.g., sloughing of cells, spawning, defecation), and the solid line represents the net concentration change based on summation of production and losses.

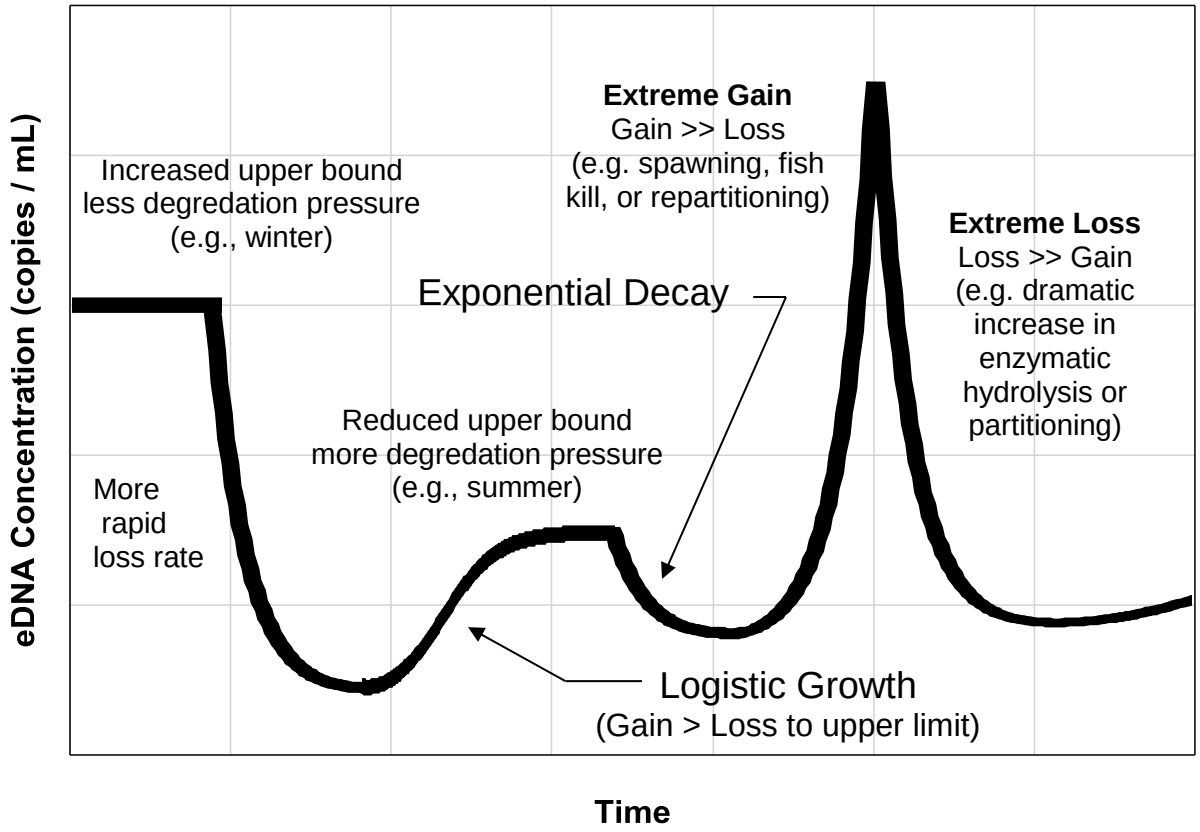

Figure 14. Proposed theoretical patterns in eDNA concentration over time.
Observed concentrations are a function of variable gains and losses through time. In steady state natural systems, eDNA concentration is expected to have an upper limit that is governed by physical loss constraints. The line represents net concentration after exponential losses due to physical and biological factors (e.g., sinking, sorption, photodegradation, hydrolysis by enzymes) and logistic growth by production (e.g., sloughing of cells, spawning, defecation). When losses > gains, there would be a decline in concentration, and where gains > losses, there would be increases to higher maximum concentrations. Extremes in either losses (e.g., light, temperature, or microbial action spikes) or gains (e.g., fish kills) in eDNA should result in relatively rapid changes.

CONCLUSIONS AND IMPLICATIONS FOR FURTHER RESEARCH

Based on the data compiled from this study, water quality of co-located tank mesocosms at the KU Field Station remains within expected norms for lotic and lentic ecosystems of eastern Kansas, and does not differ meaningfully among tanks over time. Topeka shiners housed in these tanks exhibited both 90% survival and significant growth over the course of a year, feeding solely on the phytoplankton, zooplankton, and other organic materials present in the aged well water reservoirs that supply the facility. Mean growth was larger in tanks with densities of 20 fish per $10m^3$ tank than in densities 40 or 80, but total weight was a reliable predictor of total length across a range of body sizes and densities.

Environmental DNA (eDNA) from Topeka shiners can be recovered, extracted, quantitated, and detected by PCR endpoint and quantitative assays from integrated water column samples. eDNA detection probability was found to be approximately three times higher in tanks with 80 fish, than in tanks with 40 or 20 fish. There was no observed difference in mean detection probability in tanks with fish stocked at the lower two densities. The number of detections spiked one day after stocking, presumably due to an abundance of Topeka shiner eDNA created during the stress of handling and transport, then rapidly decreased to below detection limits within 7 days. After 14 days, the number of detections in the high density tank had reached levels comparable to the initial spike, and remained at similar levels 26 days after fish stocking. However, water column concentrations of Topeka shiner eDNA did not exceed 20% of the initial spike over the course of the experiment. Accumulation trends in tanks with fish stocked at medium and low densities were less clear in the first 26 days, but did have consistent levels of detection. *N. topeka* eDNA was detected in samples 27 days after fish removal, regardless of density, but at a lower detection probability than when fish were present.

This study demonstrates that eDNA from Topeka shiners can be detected in water column samples from controlled mesocosms using qPCR assays. However, the stocking density at which the assay provided higher detection probabilities is likely higher than the density of fish in their native lotic

and lentic habitats. Also, the current qPCR assays have not been sufficiently optimized to distinguish between *N. topeka* and its closest relative, *N. stramineus*, across a broad range of eDNA concentrations. This is problematic since *N. stramineus* may co-occurs in many habitats within the Topeka shiner's range. Potential improvements to eDNA detection could be made by further marker development, which will greatly benefit from additional sequencing of the Topeka shiner's mitochondrial genome. Simultaneous use of multiple markers for *N. topeka* may also increase detection probability. Likewise, sampling of other environmental compartments (e.g., sediment or biofilms) may yield better detection, due to differences in fate and transport or recoverability of *N. topeka* eDNA Long-term monitoring to identify potential patterns would also aid in understanding the factors governing eDNA concentration, and could potentially provide insight into seasonal phenomena of interest (e.g., spawning). Finally, development of methods with lower detection limits for target eDNA quantification would provide additional information to more reliably determine not only the probability of detection/nondetection, but also the concentration of *N. topeka* eDNA. More reliable data for eDNA concentrations observed under field conditions would allow for potential quantitative analysis with other variables of interest, including questions of environmental fate (e.g., degradation rates) and transport (e.g., mass balance approaches).

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CHAPTER 4: Partitioning and degradation of eDNA from Bigheaded Carps (*Hypophthalmichthys* spp.) in earthen ponds

INTRODUCTION

Organisms shed DNA into environment, and with the rapid advancement of molecular methods for collection, extraction, amplification, sequencing, and interpretation of environmental DNA (eDNA) concentrations, applications for molecular monitoring of vertebrates are becoming more widespread. Studies have shown, for example, that fish (Takahara et al. 2012, Thomsen et al. 2012, Klymus et al. 2015) and amphibian (Goldberg et al. 2011, Thomsen et al. 2012) water column eDNA concentrations under controlled conditions are related to the biomass of source organisms present in the system, and a field study on common carp showed that water column eDNA concentrations were highest in areas where the carp spent the most time (Eichmiller et al. 2014). In a recent collection of eDNA papers focused on conservation biology, Goldberg et al. (2015) raised questions about understanding the fate and transport of vertebrate eDNA in aquatic environments, especially with respect to ecological conditions that may affect measurements by either increasing the noise (e.g., variable production, variable degradation, patchy distribution of eDNA) or decreasing the signal (e.g., inhibition).

Saba and Steinberg (2012) recently documented the rapid sinking of large amounts of fish ejecta, representing a significant mode of potential transport of genetic material from one environmental compartment (water column) to another (sediment). Since > 95% of extracellular DNA in sediments may be attached to the soil matrix (Dell'Anno et al. 2002) and since sediment particles (Vanoni 2006), flocs of fish waste (Law et al. 2014), and cellular material (Leff et al. 1992, Jamieson et al. 2005) can all be resuspended when cohesive sediments are exposed to sufficient to shear stress, transport of eDNA between the water column and surficial sediments are likely important mechanisms affecting eDNA concentrations of fish and other aquatic vertebrates.

In addition to sediment transport by naturally occurring physical phenomena such as wave action and downstream flow (Law et al. 2014), fish can also regulate sediment processes by bioturbation and alteration of the structure of benthic habitats (Holmlund and Hammer 1999). In flowing water, macrophytes have been shown to reduce flow velocities and increase sediment deposition (Schulz et al. 2003), and sediment resuspension within plant beds has been observed to be less than half that of resuspension in open areas (Horppila and Nurminen 2003). However, high densities of grass carp (*Ctenopharyngodon idella*) can result in complete elimination of submerged macrophytes (Leslie et al. 1987); and once macrophytes have been depleted below threshold levels, sediment resuspension may increase significantly (Li et al. 2008).

Given the potential link between water column and sediment concentrations of fish eDNA, I hypothesized that in relatively quiescent lentic systems with limited inflow and outflow, sediment concentrations of fish eDNA would be higher than fish eDNA concentrations in the overlying water column and that increased resuspension in those systems would result in increased water column concentrations of fish eDNA. I also hypothesized that higher amounts of fish would yield higher concentrations of fish eDNA in both the water column and in surficial sediments.

In addition to transport, eDNA undergoes degradation in aquatic systems by three mechanisms: physical disruption by UV light and temperature, chemical disruption by depurination, and biological disruption by enzymatic hydrolysis (Lindahl 1993). Degradation in marine sediments has been shown to occur at higher rates than in overlying water (Dell'Anno and Corinaldesi 2004). Because DNA degrading enzymes are generally rate limited by substrate availability (Demaneche et al. 2001), and because sediments may have higher eDNA concentrations than overlying water (Dell'Anno et al. 2002, Dell'Anno and Danovaro 2005), increased enzyme activity is the likely reason for this observation. Presumably, similar behavior occurs in freshwater systems, so higher fish eDNA concentrations should coincide with higher degradation rates. At the same time, extracellular DNA in aquatic systems can attach to clay particles in sediment (Demaneche et al. 2001, Theng 2012), and (Dell'Anno et al. 2002) observed that

>50% of sediment extracellular DNA may be protected from deoxyribonuclease (DNase) hydrolysis by such attachment. Clay particles have been shown to prevent decay of DNA by protection of DNA from enzymatic hydrolysis by both direct binding of DNA and competitive binding of enzymes (Demaneche et al. 2001). Resuspension and mixing may also promote adsorption of DNA to clay sediments. Therefore, if sediments are composed at least in part by clays, higher resuspension should be associated with lower degradation rates.

In order to test these hypotheses on fish eDNA concentrations in sediment, a two-part study design was developed to examine the first three hypotheses on the transport and the second two hypotheses on the differential fates of fish eDNA in aquatic systems.

METHODS

Study Design

Two sediment eDNA studies were undertaken simultaneously. The goal of the first was to characterize *Hypophthalmichthys* spp. eDNA concentrations in aquatic sediments of ponds where the fish had been continuously present for an extended period of time. Water column eDNA samples from the same ponds were also collected for comparison of the relative partitioning of eDNA between water and sediment. Findings of the first study have been published (Turner et al. 2015) and are included as Appendix A. The goal of the second study was to examine the rate of degradation of eDNA in freshwater sediments.

Study Site

As part of a larger study on Asian carp by the University of Notre Dame, fish were introduced to 10 man-made earthen ponds at the University of Kansas Field Station (KUFS) near Lawrence, KS (USA) (Figure 1). The ponds are approximately 450 m^3, with a 2:1 side slope and a maximum depth of 3 m.

The ponds were filled simultaneously with surface-aged well water from an onsite reservoir containing no fish. Once filled, the valves were closed, and the ponds isolated. No Asian carp had been located at the facility prior to the study, and sediments from all ponds tested negative for Asian carp prior to filling. Pond substrates were generally characterized prior to this study as Silty Clay Loam (approximately 14% sand, 48% silt, and 38% clay) with bulk density of about 1 g/cm^3, cation exchange capacity of approximately 22 meq/100 g, maximum water holding capacity of about 53 g/100 g, and organic carbon content of approximately 2%.

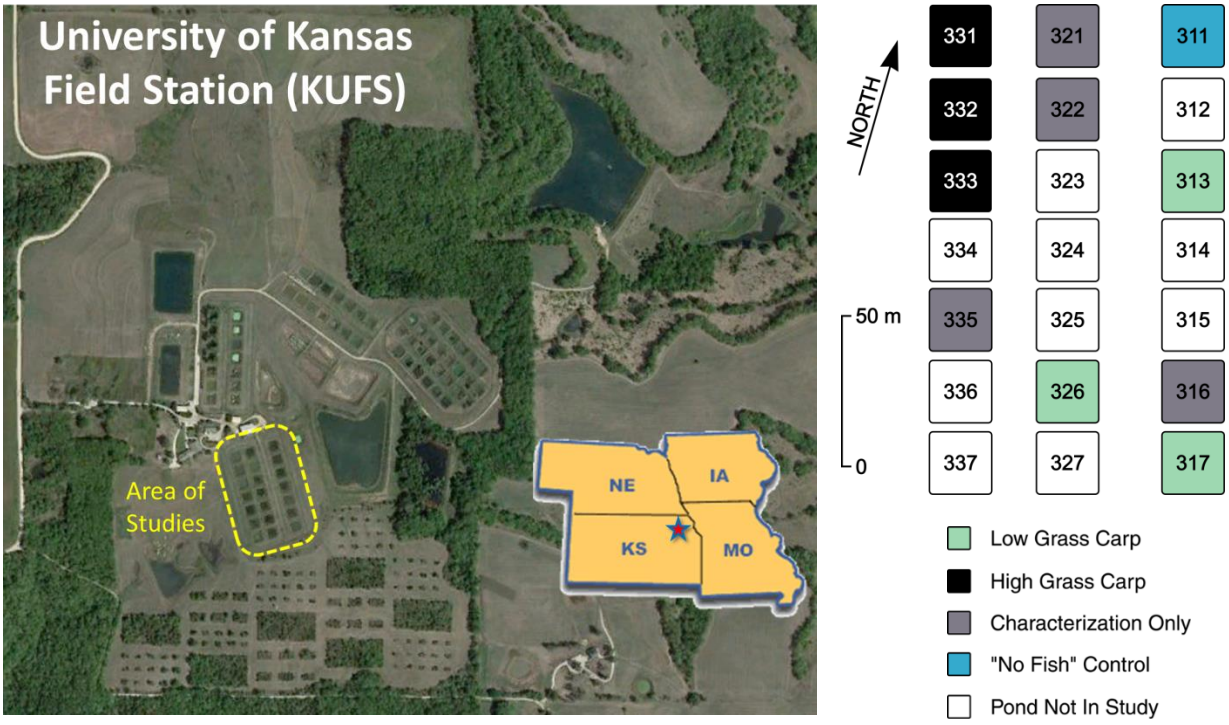


Figure 1. Location map, pond layout, and sampling design.
Ponds were stocked at various rates with fish of multiple species. The "No Fish" control pond was not stocked with fish, and no fish were present in that pond throughout the study. The six Low and High Grass Carp ponds were used in for the sediment degradation study, with Low and High referring to the relative abundance of grass carp in these ponds. All 11 ponds (3 Low Grass Carp, 3 High Grass Carp, 4 Characterization Only, and 1 No Fish control) were used for the characterization study.

A community of fish including Bighead carp (*Hypophthalmichthys nobilis*), Silver carp (*Hypophthalmichthys molitrix*), Redear sunfish (*Lepomis microlophus*), Bluegill sunfish (*Lepomis macrochirus*), White crappie (*Pomoxis annualris*), common carp (*Cyprinus carpio*) and grass carp (*Ctenopharyngodon idella*) were stocked at varying densities (Table 1). Since the assay used to quantify environmental DNA of Asian carp targets the genus *Hypophthalmichthys*, both species will be referred to collectively as bigheaded carps for the purposes of this study. All fish were stocked and handled in accordance with protocol 211-01 approved for field research on live vertebrates by the Institutional Animal Care and Use Committee of the University of Kansas. One pond (311) was not stocked and served as a no fish control. All ponds had been stocked with at least one *Hypophthalmichthys* spp. individual by 30 May 2012, and the fish remained in place until sampling for this study began 14 Oct 2012. In the interim, water temperature was recorded *in situ* at 15 minute intervals via two submerged datalogging HOBO probes (Onset Corp., USA), and additional water quality parameters (i.e., dissolved oxygen, pH, specific conductivity, turbidity, and 5-day biological oxygen demand) were measured on a regular basis. Fish mortalities were removed from the ponds as quickly as possible, carcasses were carefully contained and disposed of, and all equipment used in transport and handling of the carcasses was thoroughly decontaminated with 20% bleach to avoid cross-contamination of eDNA from the high copy numbers associated with whole fish tissues. Only one bigheadded carp was stocked in pond 321, and it died and was removed 132 days prior to sampling for these experiments.

Additional field samples were collected from three natural river sites: the Wabash River (West Lafayette, IN, USA), the Kansas River (near Desoto, KS, USA), and the Wakarusa River below Clinton Reservoir (near Lawrence, KS, USA).

Sample Collection and Preservation

Both water samples and sediment samples were collected as described in (Turner et al. 2015). Three 15 mL surface water samples were collected in sterile 50mL centrifuge tubes using new sterile

gloves for each pond and field site. 33.5 mL of 100% EtOH and 1.5 mL of 3M sodium acetate were added to each tube (Ficetola et al. 2008), and the tubes were stored on ice for less than 120 minutes until transfer to a -20 °C freezer. A field blank (15 mL of tap water) was collected prior to field sampling and placed in the cooler used for transporting the pond samples. The field blank accompanied the pond samples throughout processing as a quality control. After freezing, samples were transferred on dry ice overnight to the University of Notre Dame and stored at -80 °C until extraction. Water samples were collected prior to sediment samples to avoid potential resuspension or other potential contamination associated with the sediment collection process.

Sediment samples were collected by adaptation of a method originally used for retrieval of diatom frustules from surficial sediments (US Environmental Protection Agency 2007). Using a hand corer equipped with sufficient weight to pierce the pond sediment, the top 2 cm of pond sediments were collected via a stage and sectioning apparatus fitted to the core liner tube (Figure 2) (Glew and Smol 2001). Cores were collected along representative transects in both pond and field sites. Two sets of cores were taken: an initial characterization set and a degradation study set.

(a) **(b)**

Figure 2. Sediment coring apparatus including (a) corer, nose piece, liner tube, egg shell sediment retainer, and stopper and (b) sediment sectioning stage and sectioner used for collecting surficial sediments.

Table 1. Established fish community parameters.

Time	Parameter	Pond									
		313	**316**	**317**	**321**	**322**	**326**	**331**	**332**	**333**	**335**
Initial Stocking	Count Density of BC. (count / L)	0.004	0.048	0.036	0.002	0.002	0.102	0.011	0.018	0.024	0.029
	Count Density of GC (count / L)	0.008	0.019	0.025	0.009	0.017	0.007	0.052	0.032	0.031	0.014
	Count Density of non BC. (count / L)	0.025	0.038	0.043	0.026	0.033	0.024	0.073	0.048	0.046	0.029
	Biomass of BC (g / L)	0.6	6.9	4.6	0.8	1	15.8	1.4	3.5	3.7	2.3
	Biomass of GC (g / L)	2.3	4.9	6.7	2	4.3	2.5	9.5	7.5	8.4	1.8
	Biomass of non BC. (g / L)	4.1	7	8.5	3.7	6.2	4.3	11.5	9.1	9.6	3.4
	Count Ratio of GC to BC	2.0	0.40	0.69	4.50	8.5	0.07	4.7	1.8	1.3	0.48
	Biomass Ratio of GC to BC	3.8	0.71	1.5	2.5	4.3	0.16	6.8	2.1	2.3	0.78
Final Harvest	Count Density of BC. (count / L)	0.008	0.084	0.056	0	0.005	0.141	0.014	0.04	0.07	0.055
	Count Density of GC (count / L)	0.016	0.032	0.034	0.021	0.042	0.016	0.099	0.087	0.113	0.044
	Count Density of non BC. (count / L)	0.036	0.065	0.056	0.048	0.058	0.042	0.099	0.113	0.139	0.088
	Biomass of BC (g / L)	7.5	48	40.8	0	10.3	71	23.9	71.4	100.9	65.3
	Biomass of GC (g / L)	36.4	53.4	57.4	46.9	76.5	43	75.8	112.3	159.6	78.5
	Biomass of non BC. (g / L)	40.8	60.8	63.6	54.3	80.5	50.3	75.8	117.3	164.1	90.2
	Count Ratio of GC to BC	2.0	0.38	0.61	-	8.4	0.11	7.1	2.2	1.6	0.80
	Biomass Ratio of GC to BC	4.9	1.1	1.4	-	7.4	0.61	3.2	1.6	1.6	1.2

BC indicates bigheaded carp (both species of *Hypophthalmichthys*)
GC indicates grass carp (*Ctenopharyngodon idella*)
non BC indicates fish other than bigheaded carp, including Redear sunfish (*Lepomis microlophus*), Bluegill sunfish (*Lepomis macrochirus*), White crappie (*Pomoxis annualris*), common carp (*Cyprinus carpio*) and grass carp (*Ctenopharyngodon idella*)
Count ratio of GC to BC indicates the ratio of count density of grass carp to count density of bigheaded carp
Biomass ratio of GC to BC indicates the ratio of biomass of grass carp to biomass of bigheaded carp

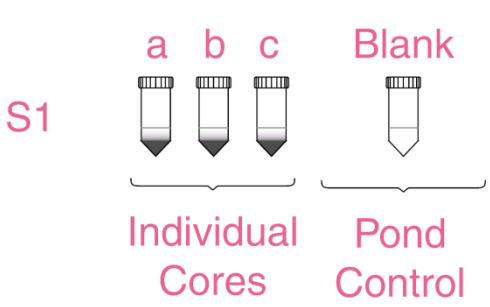
Initial Characterization Study Samples

To get an idea of the general sediment concentrations of eDNA, we collected 3 cores along a single transect in each of 11 ponds (Figure 3, Table 2). Single transects of 3, 3, and 2 cores were also taken at three Midwestern river sites on the Wabash River, Kansas River, and Wakarusa River, respectively. Cores were collected primarily from boats, with samples from the Kansas River taken by hand using sterile centrifuge tubes from the littoral zone due to a lack of cohesive sediments. For each core, a 5mL wet sediment sample was taken from the top 2cm and placed in a 50mL centrifuge tube containing 10mL of sterile cetyl trimethyl ammonium bromide (CTAB) (Coyne et al. 2001, Coyne et al. 2006). Wet sediment weights were calculated by subtracting the sediment-filled tube weight from the weight of the tube with CTAB only. Filled and fixed tubes were placed on ice and transferred to a -20 °C freezer within 120 minutes of sampling. After freezing, the sediment tubes were shipped overnight on dry ice to the University of Notre Dame and stored at -80 °C until extraction. River site samples were handled in a similar manner, with the exception of the Kansas and Wakarusa sediment samples, which were placed in sterile centrifuge tubes without CTAB, held on ice for less than 120 minutes, frozen at -20 °C, and then shipped overnight on dry ice to the University of Notre Dame. Samples were thawed, weighed, preserved with CTAB there.

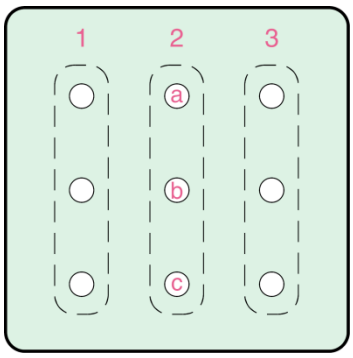
Degradation Study Samples

To carry out a time series degradation study, a larger volume of sediment material was required. For this study, we collected along three transects in each of 6 ponds (Figure 1, Figure 3, Table 2). For each transect, we collected the top 2 cm from each of three cores, then combined and mixed them thoroughly to form a composite transect sample. From each transect composite, (7) 5 mL subsamples were taken for time series analysis. Each 5 mL subsample of wet sediment was placed in a sterile 50 mL centrifuge tube, yielding 7 subsamples per composite and 21 subsamples per pond. One subsample was randomly selected from each composite set, preserved with 10 mL of CTAB, placed on ice for less

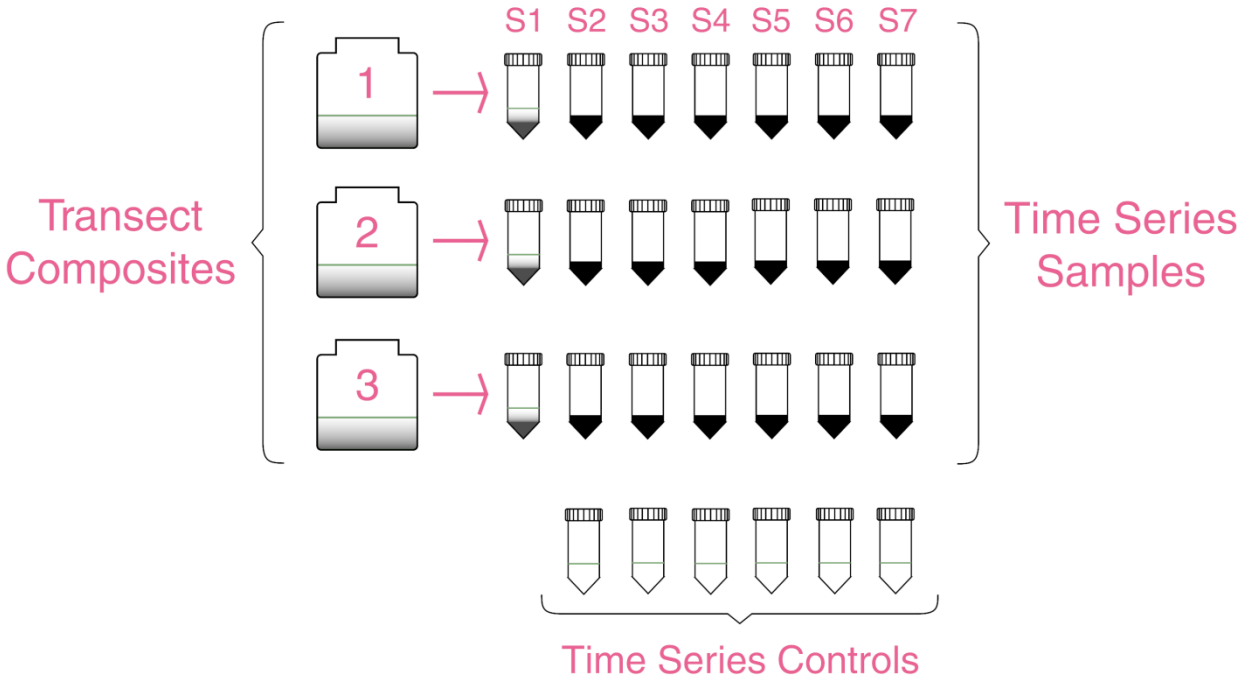
than 120 minutes, and then weighed and frozen at -20 °C. Weight of wet sediment was determined by subtraction as above. The remaining time series samples were held in the KUFS Armitage laboratory at ambient temperature until scheduled preservation (Table 3). At the scheduled time, 1 subsample from each composite (i.e., 3 samples per pond) were randomly selected, preserved with 10 mL CTAB, and weighed to determine wet weight of sediment. Preserved samples were then frozen at -20 °C and held until overnight transport on dry ice to the University of Notre Dame for extraction. This process was repeated for all seven sampling events (0, 0.5, 1, 2, 5, 10, and 120 days, respectively).



(a) Characterization Study Samples



(c) Sampling schematic for event S1



(b) Degradation Study Samples

Figure 3. Schematic diagrams for sediment eDNA samples collected for (a) the characterization study, (b) the degradation study, and (c) the general layout of sediment sample collection. Three samples were taken and preserved at the first sampling event (S1) for both the characterization study (3 individual cores) and the degradation study (transect composites). For degradation study ponds, the characterization study individual core samples were taken from one of the transects prior to compositing the sample, allowing for within and between transect investigation. Controls were 10 mL of tap water preserved in 10 mL of CTAB solution. The pond control also serves as the S1 time series control.

Table 2. Sampling regime for the sediment eDNA concentration studies.

Purpose	Parameter	Sampling Location													
		311	313	316	317	321	322	326	331	332	333	335	Wabash R.	Kansas R.	Wakarusa R.
Characterization Study	Transects per Location	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Cores per Transect	3	3	3	3	3	3	3	3	3	3	3	3	3	2
	Samples per Core	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Samples per Location	3	3	3	3	3	3	3	3	3	3	3	3	3	2
Degradation Study	Transect Composites	3	3	-	3	-	-	3	3	3	3	-	-	-	-
	Subsamples per Composite	1	7	-	7	-	-	7	7	7	7	-	-	-	-
	Subsamples per Location	3	21	-	21	-	-	21	21	21	21	-	-	-	-

Table 3. Sample preservation schedule for sediment eDNA degradation study.

Sampling Event	S1	S2	S3	S4	S5	S6	S7
Days Until Preservation	0	0.5	1	2	5	10	120

For both sets of sediment samples, cores were taken with sterile gloves using WildCo hand corers. Core liner tubes, corers, sediment stages, sediment samplers, and ropes were sterilized after each core by a four step process: first, by removal of large bits of sediment with native water; next, by immersion and scrubbing in hot, soapy water; then followed by immersion in a 10% bleach and tap water solution for 10 minutes, and then again followed by a tap water rinse. After all cores for a given pond or site were collected and the coring apparatus cleaned, bleached, and rinsed, a field blank was taken by collection of 15mL of rinse water in a sterile 50mL centrifuge tube containing 10mL of sterile CTAB. Coring apparatus controls were also collected. Once each coring apparatus (corer, liner tube, and nosepiece) was cleaned and re-assembled, 5 mL of tap water was flushed through and collected in a 50 mL tube containing 10 mL of sterile CTAB. As with the other samples, CTAB-preserved field and coring apparatus blanks were placed immediately on ice in the dark, held for less than 120 minutes, then frozen at -20 °C. Once frozen, they were shipped overnight on dry ice to Notre Dame and held at -80 °C for processing with the other samples.

Laboratory Methods

Extractions and PCR setup were performed in a strictly pre-PCR laboratory separate from the post-PCR laboratory. Experimental controls were included throughout the experiment with field blanks, corer apparatus controls, extraction blanks (5 or 10mL of autoclaved reverse osmosis water treated as a sample), no template controls and standard copy number curves for negative and positive amplification controls, respectively, and internal positive controls for inhibition.

DNA Extraction

We modified previously published CTAB protocols (Coyne et al. 2001, Coyne et al. 2005, Coyne et al. 2006) for chloroform and isoamyl alcohol extraction of eDNA from water samples. A detailed description of the extraction protocol appears in Appendix A. DNA was initially precipitated

from water using EtOH and sodium acetate (Ficetola et al. 2008), followed by centrifugation at 3220 x *g* at 6 °C for 35 minutes. The supernatant was poured off, and after the pellet was air dried for 5 minutes, 700 uL of CTAB were added. The CTAB and pellet were incubated at 60 °C for 10 minutes, then the sample contents were transferred to a microcentrifuge tube containing 700uL of 24 : 1 chlorform : isoamyl alcohol. Samples were mixed for exactly 5 minutes, then centrifuged at 3220 xg for 15 minutes at room temperature. The aqueous phase was then transferred to a new microcentrifuge tube, and an equal volume of ice-cold isopropanol and a half volume of 5M NaCl were added. Samples were then incubated at -20 °C for 1 hour to 2 days. After incubation, samples were again centrifuged at 3220 xg for 15 minutes at room temperature. The supernatant was decanted, the pellet was rinsed with 70% ethanol for 2 minutes, and the ethanol was poured off and the pellet was allowed to air dry completely. The resultant aqueous eDNA pellet was eluted in 100 uL of 1x TE Low EDTA Buffer (USB Corporation, USA) and stored at 4 °C for assay by qPCR.

Sediment samples were extracted by similarly modified methods. Samples were thawed, incubated at 60 °C for 10 minutes, and then 15mL of 24 : 1 chlorform : isoamyl alcohol were added and the sample mixed for exactly 5 minutes. After another 15 minute centrifuge at room temperature for 15 minutes, the aqueous phase was transferred to a new sterile 50 mL tube and an equal volume of ice cold isapropanol and a half volume of 5M NaCl were added. Samples were then incubated at -20 °C for 1 hour to 2 days. After incubation, samples were again centrifuged at 3220 xg for 15 minutes at room temperature. The supernatant was decanted, the pellet was rinsed with 70% ethanol for 2 minutes, and the ethanol was poured off and the pellet was allowed to air dry completely. Sediment eDNA was eluted in 1000 uL of 1x TE Low EDTA Buffer.

Resultant water and sediment eDNA extracts were quantitated using Qubit dsDNA High Sensitivity assay (Life Technologies, USA).

Every extract was tested for inhibition using a Universal Exogenous qPCR Positive Control for TaqMan Assays kit (Eurogentec, USA). The internal positive control (IPC) assay was run in duplex with the *Hypophthalmichthys* assay for quantification of bigheaded carp eDNA. By comparing the average quantification cycle (Cq) of the IPC in the standard curve and no template controls on each plate (the expected IPC Cq) to the Cq of the IPC in each sample well (the observed IPC Cq), we were able to calculate an IPC delta Cq value (IPC expected Cq - IPC observed Cq). Since the maximum range in IPC Cq values of the standard curve and no template controls was 2.8, a threshold change of 3 cycles was used to identify inhibition (Champlot et al. 2010). Any samples with IPC delta Cq values larger than 3 were treated as inhibited.

Based on inhibition testing of a subset of sediment samples using this IPC protocol, we further purified 200 uL of sediment eDNA extract from every sample using a OneStep Inhibitor Removal Kit (Zymo Research, USA). Subsequent testing of the OneStep purified sediment extracts showed no evidence of continued inhibition after the purification.

DNA Amplification

We used a Minor-Groove Binding hydrolysis probe (Turner et al. 2014) to amplify a 100 bp section of the mitochondrial control region (D-loop) of *Hypophthalmichthys* spp. Amplification was performed on an Eppendorf Mastercycler ep realplex2 S thermocycler (Eppendorf, USA) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and 55 cycles of 95 °C for 15s followed by 60 °C for 1 min. 10 uL of TaqMan Environmental Master Mix 2.0 (Life Technologies, USA), 300 nM each of the forward and reverse primer, 200 nM of the probe, and 4 uL of the template were combined in 20 uL reactions. We combined eDNA template, primers, probe, and mastermix in six reaction aliquots in a sterile tubes to minimize variation between technical replicates (Barbour et al. 1999), then used an electronic autopipetter (Eppendorf, USA) to load the qPCR plate. qPCR setup was performed in an AirClean 600 dead air box with ultraviolet light (AirClean Systems, USA) using low bind tubes and low

bind aerosol barrier pipette tips. Each qPCR plate contained 2 no template controls and a five-point copy number standard curve made from the complete 1022 bp D-loop PCR amplicon from tissue-derived Silver Carp DNA. The standard curve ranged from 3×10^5 copies per reaction to 3 copies per reaction. The MGB assay used in this study has a 95% detection limit of 30 copies per reaction.

Aqueos and sediment eDNA concentrations were calculated by setting non-detect reactions to zero, then taking the mean of sextuplicate technical reactions rounded to the next largest integer. Biomass concentrations of fish at the time of sample collection were based on linear interpolation of growth between stocking and harvest, divided by pond volumes calculated from water depths measured on the day of sampling.

Statistical analyses for the characterization study were performed using R 3.0.1 (R Core Team 2015) and for the degradation study using NCSS 9 (Hintze 2013).

RESULTS

A range of grass carp (*Ctenopharyngodon idella*) and bigheaded carp densities were stocked across the experimental ponds (Table 4). Over the course of the 137 days with fish communities in place prior to the start of this study, visual evidence of physical changes between ponds with relatively high grass carp densities and those with relatively low grass carp densities became apparent (Figure 4). We observed multiple lines of evidence of bioturbation effects from grass carp, including changes in water quality and direct physical disturbance of substrates during daily observation of the ponds. In ponds where the ratio of grass carp biomass to bigheaded carp biomass was 1 or more (i.e., equal or greater biomass of grass carp than bigheaded carp biomass), extreme turbidity events were more frequent (Figure 5), BOD5 was significantly higher (Figure 6), dissolved oxygen had a larger range (Figure 7), suspended chlorophyll *a* was significantly higher (Figure 8), and mean pH was lower by about 0.25 units (Figure 9). In addition, macrophytes were either largely or completely eliminated from ponds 331, 332, and 333, and episodic bluegreen algal blooms were observed.

Table 4. Bigheaded carp (*Hypophthalmichthys* spp.) and grass carp (*Ctenopharyngodon idella*) biomass concentration and count by pond.
GC:Hypop Biomass Ratio is the biomass of grass carp divided by the biomass of bigheaded carp. GC:BC Biomass ratio is the ratio of the grass carp biomass to the bigheaded carp biomass. Green shading indicates ponds with GC:BC Biomass Ratio greater than 1.

Pond	Bigheaded Carp (*Hypophthalmichthys* spp.)			Grass Carp			GC:BC Biomass Ratio
	Biomass (g/L)	Average Biomass per Fish (g)	Count	Biomass (g/L)	Average Biomass per Fish (g)	Count	
313	0.00722	892	2	0.0263	2162	3	3.6
316	0.0460	552	13	0.0111	1728	1	0.24
317	0.0405	708	13	0.0123	2785	1	0.30
322	0.00968	1887	1	0.0716	1745	8	7.4
326	0.0660	492	27	0.0394	2643	3	0.060
331	0.0232	1698	3	0.0742	739	22	3.2
332	0.0664	1716	6	0.0901	1276	11	1.4
333	0.0962	1395	8	0.109	1266	10	1.1
335	0.0646	1137	5	0.0656	1932	3	1.0

(a) Pond 331 (Grass Carp Biomass : Bigheaded Carp Biomass Ratio = 3.2)

(b) Pond 326 (Grass Carp Biomass : Bigheaded Carp Biomass Ratio = 0.6)

Figure 4. Typical conditions of ponds with grass carp in (a) relatively high and (b) relatively low densities.
Ponds were denoted as high grass carp density if the grass carp biomass to bigheaded carp biomass ratio was 1 or greater. Ponds designated as low grass carp density ponds had the same ratio less than one. Note the lack of aquatic macrophytes and algal bloom present in high ratio pond versus the dominant macrophyte coverage and lack of algal bloom in the low ratio pond.

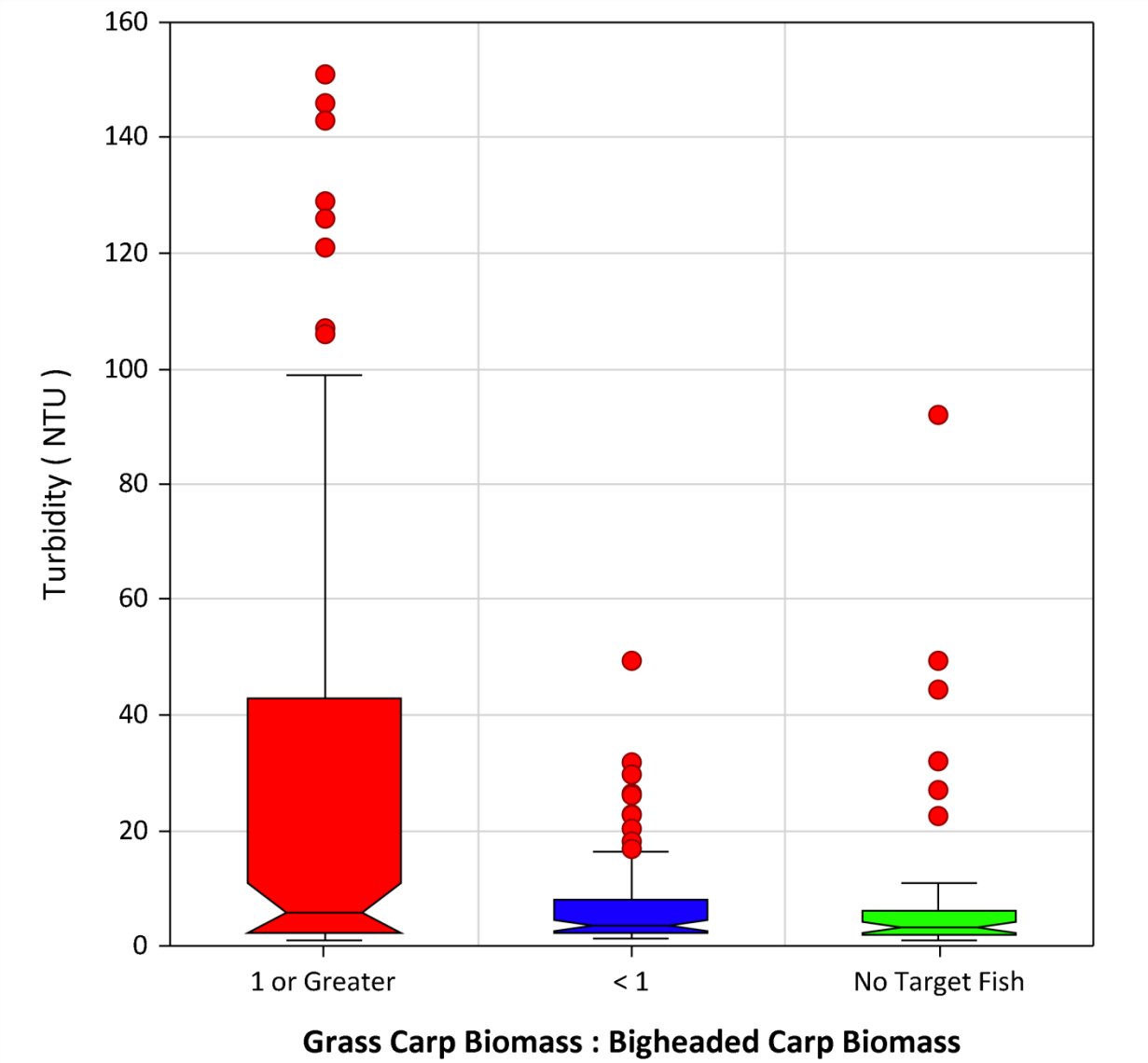


Figure 5. Boxplot of turbidity by biomass ratio of grass carp to bigheaded carps.
Values represent the distribution of in situ measurements taken between 1 and 3 times per week from May through October of 2012. Group designations indicate groups based on the ratio of grass carp biomass to bigheaded carp biomass when sediment sampling occurred in October 2012. The No Target Fish group consists of ponds that had no bigheaded carps present at the time of sediment sampling (i.e., no fish control pond 311 and pond 321).

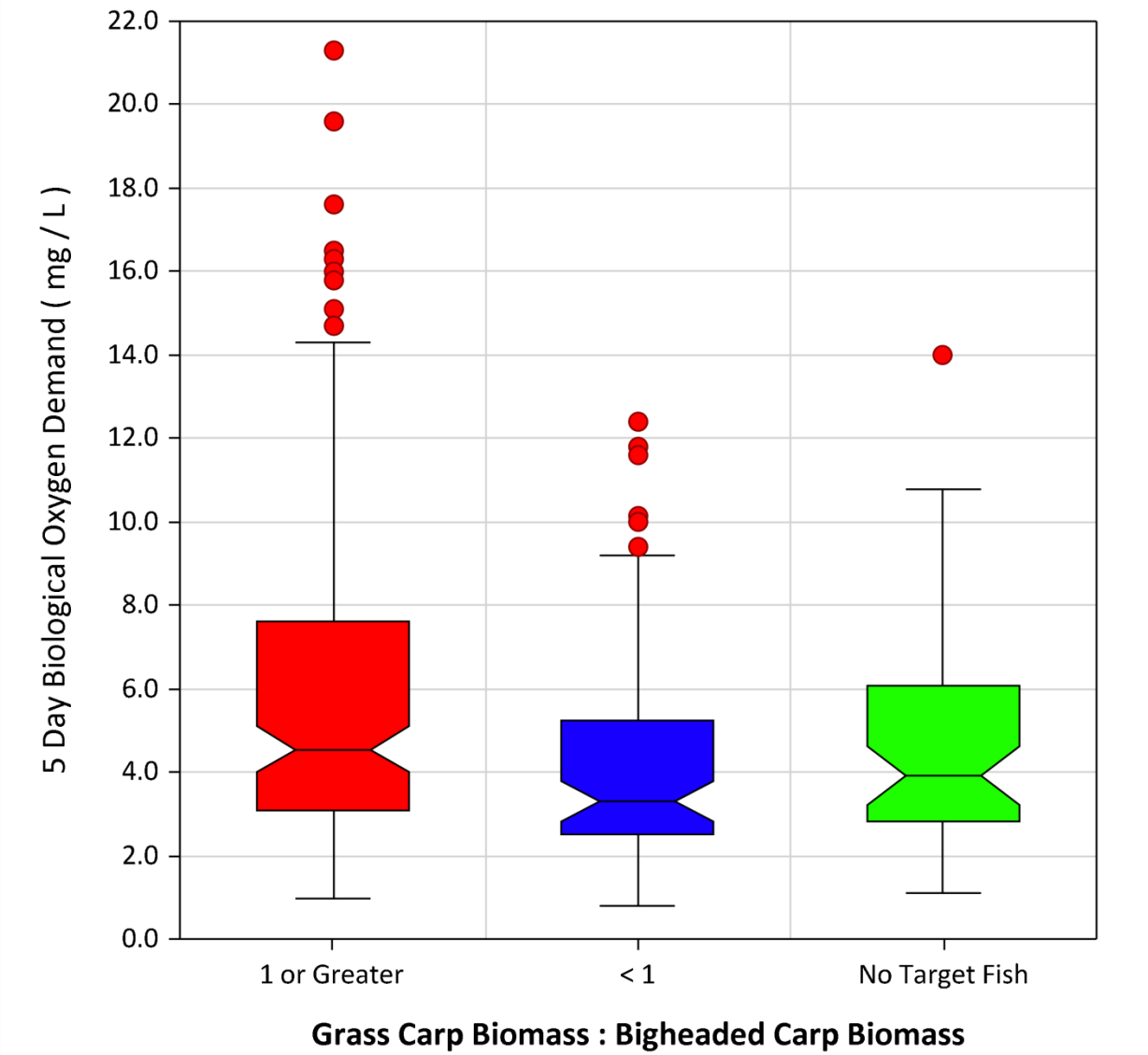


Figure 6. Boxplot of 5 day biological oxygen demand by biomass ratio of grass carp to bigheaded carps.

Values represent the distribution of in situ measurements taken between 1 and 3 times per week from May through October of 2012. Group designations indicate groups based on the ratio of grass carp biomass to bigheaded carp biomass when sediment sampling occurred in October 2012. The No Target Fish group consists of ponds that had no bigheaded carps present at the time of sediment sampling (i.e., no fish control pond 311 and pond 321).

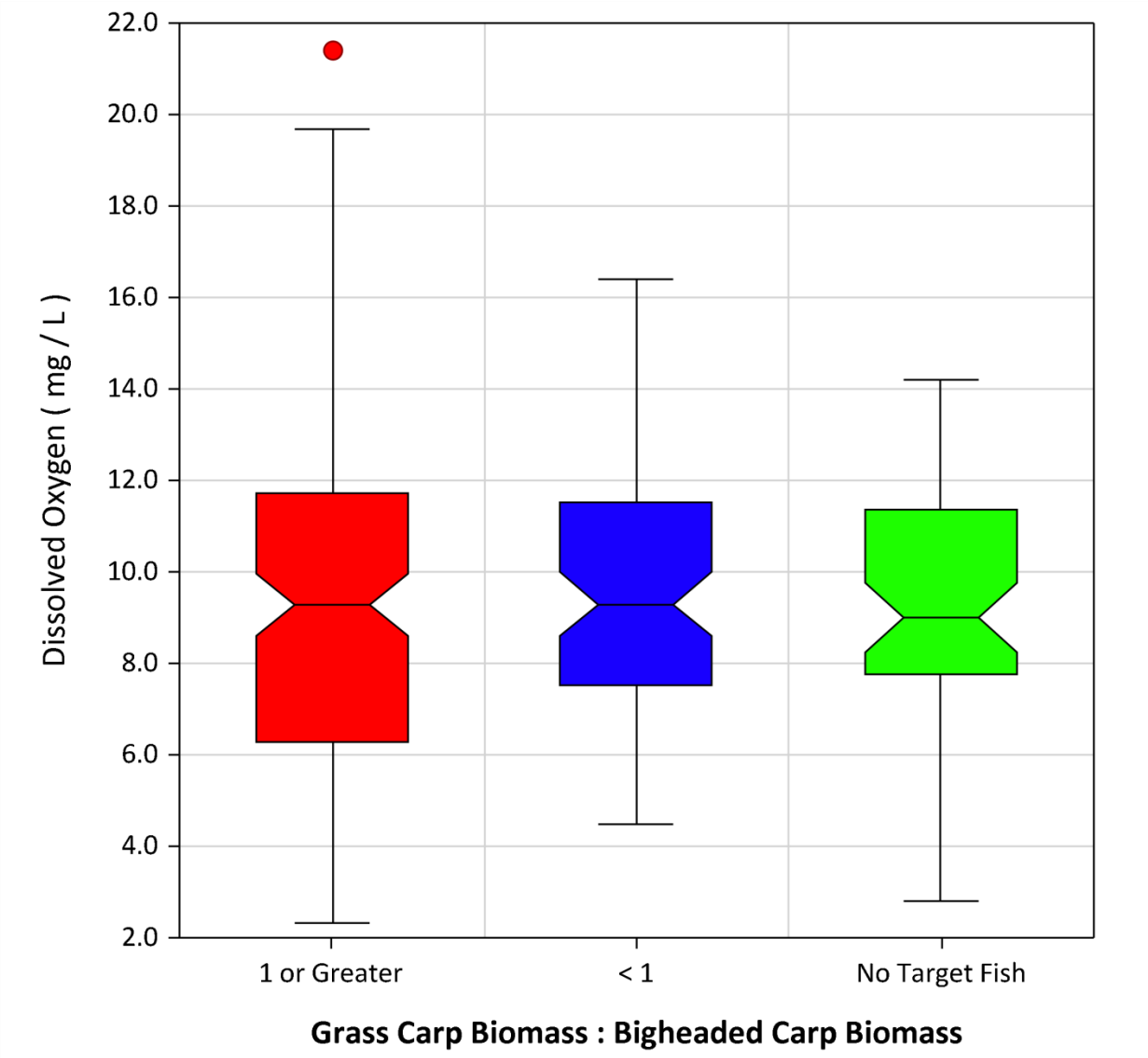


Figure 7. Boxplot of dissolved oxygen by biomass ratio of grass carp to bigheaded carps. Values represent the distribution of in situ measurements taken between 1 and 3 times per week from May through October of 2012. Group designations indicate groups based on the ratio of grass carp biomass to bigheaded carp biomass when sediment sampling occurred in October 2012. The No Target Fish group consists of ponds that had no bigheaded carps present at the time of sediment sampling (i.e., no fish control pond 311 and pond 321).

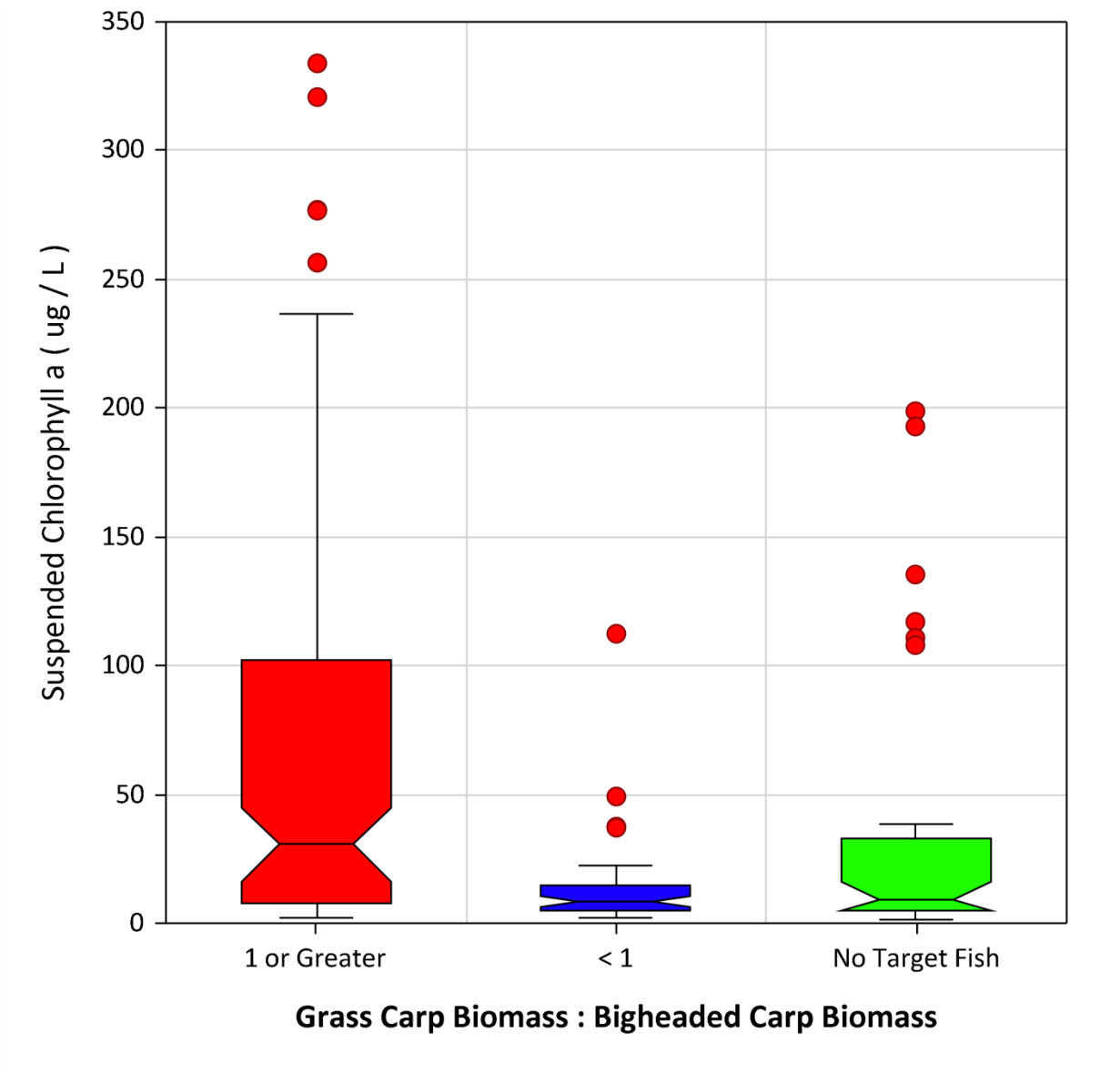


Figure 8. Boxplot of suspended chlorophyll a by biomass ratio of grass carp to bigheaded carps. Values represent the distribution of in situ measurements taken between 1 and 3 times per week from May through October of 2012. Group designations indicate groups based on the ratio of grass carp biomass to bigheaded carp biomass when sediment sampling occurred in October 2012. The No Target Fish group consists of ponds that had no bigheaded carps present at the time of sediment sampling (i.e., no fish control pond 311 and pond 321).

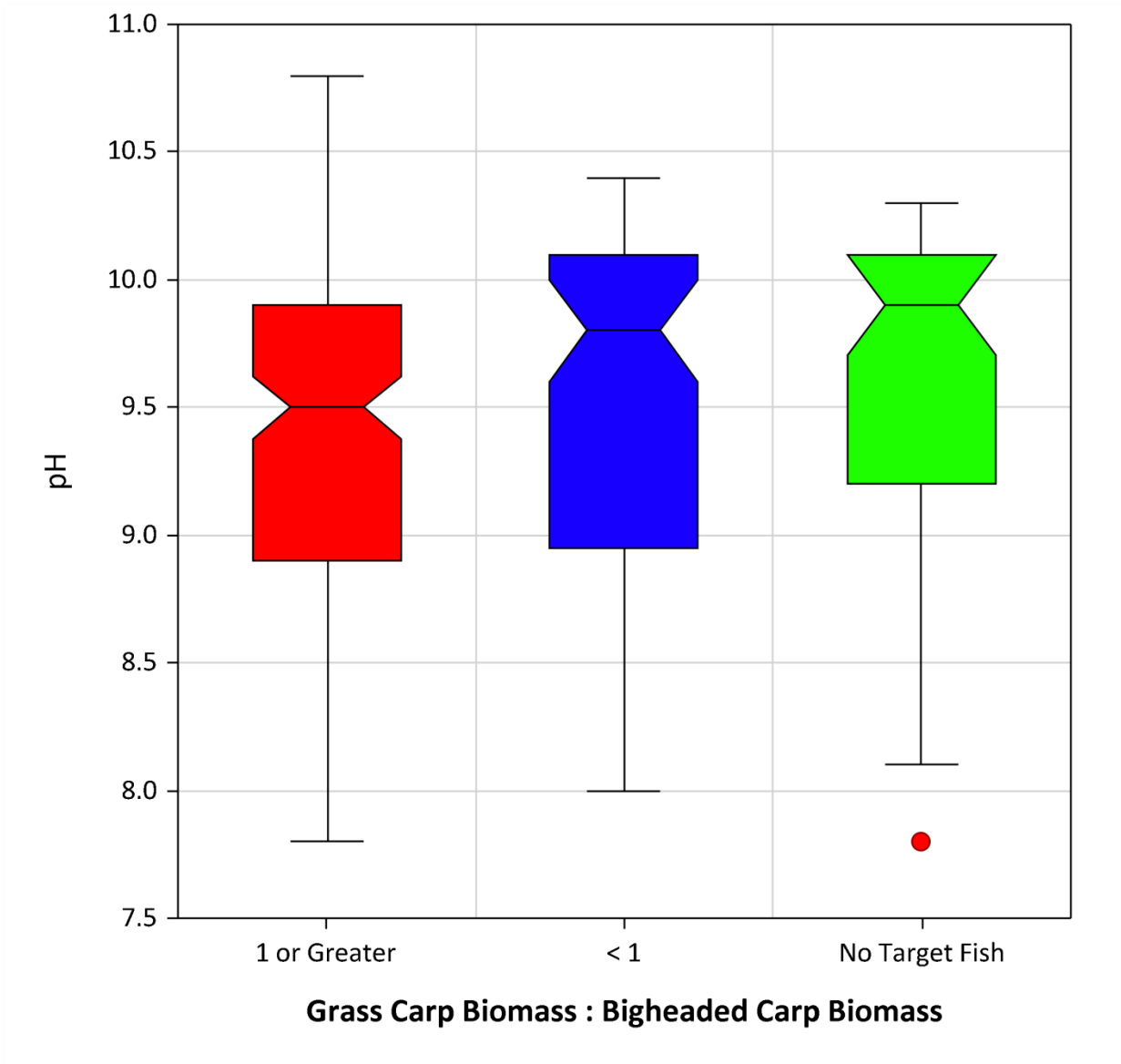


Figure 9. Boxplot of pH by biomass ratio of grass carp to bigheaded carps.
Values represent the distribution of in situ measurements taken between 1 and 3 times per week from May through October of 2012. Group designations indicate groups based on the ratio of grass carp biomass to bigheaded carp biomass when sediment sampling occurred in October 2012. The No Target Fish group consists of ponds that had no bigheaded carps present at the time of sediment sampling (i.e., no fish control pond 311 and pond 321).

Characterization Study Results

Hypophthalmichthys spp. eDNA was successfully recovered from both the water column and sediments experimental ponds and three natural river sites. The control (no fish) pond had no positive amplifications for either water column or sediment eDNA. In addition, all field blanks, extraction blanks, coring apparatus blanks, and no template controls were negative, indicating quality control procedures were sufficient to prevent contamination of samples and equipment from sample collection through qPCR amplification. Similarly, the internal positive controls confirmed that amplification of both water column and sediment samples was not limited by inhibition once the OneStep Inhibitor Removal Kit was employed.

Hypophthalmichthys spp. eDNA was detected in water samples from 8 of 10 KUFS ponds and all three river sites and in sediment samples from 10 of 10 KUFS ponds and all three river sites where the fish had either been stocked or previously observed (Figure 10, Table 5). Assuming the equivalence of 1mL and 1 g of water, sediment concentrations of bigheaded carp eDNA were significantly higher than water column concentrations (8 to 1846 times) for all sampling locations (Wilcoxon sign rank test, $p = 0.0002$). Moreover, sediment and water column eDNA concentration were positively correlated in both the experimental ponds ($p = 0.002$) and the natural river sites ($p = 0.001$). Detection probabilities ranged from 72% in water column samples to 89% in sediment samples across all sites. Since the 95% confidence intervals for these estimates overlap, there is no significant difference in detection probability between the two methods. However, bigheaded carp eDNA was detected in the sediments but not in the water column of KUFS pond 321, where the one and only stocked bigheaded carp had been removed 132 days prior to sampling (Figure 10, Table 5). A more detailed description of the characterization study and its results appears in Turner et al. (2015) and Appendix A.

A marked increase in the explanatory power of bigheaded carp biomass density ($R^2 = 0.82$) was observed in sediment eDNA concentrations for ponds with a grass carp biomass density to bigheaded

carp biomass density ratio smaller than one (i.e., relatively fewer grass carp than bigheaded carp), but not for ratios larger than one (i.e., relatively more grass carp). No equivalent grass carp density effects were observed for water column eDNA concentrations.

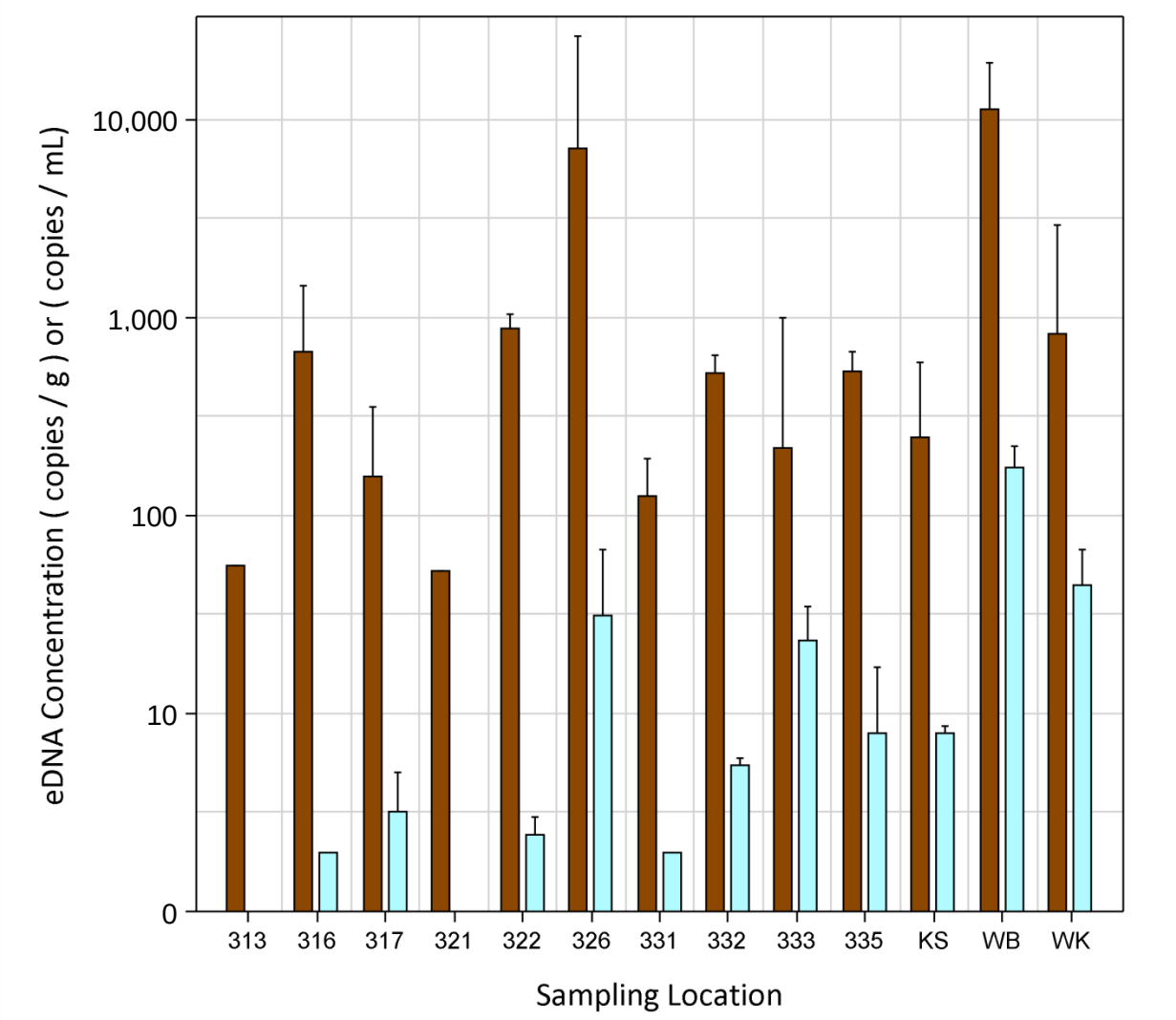


Figure 10. Water column and sediment concentrations of Hypophthalmychthys eDNA by sampling location.
Numbers indicate KUFS ponds, KS indicates Kansas River, WB indicates Wabash River, and WK indicates Wakarusa River. Dark bars represent sediment concentrations (copies / g) and light bars represent water column concentrations (copies / mL). Error bars show 1 standard error of the mean.

Table 5. Water column eDNA concentration versus Sediment eDNA concentration for all sites.

Sampling Site	Sediment [eDNA] (copies / g)					Water Column [eDNA] (copies / mL)					Sediment [eDNA]: Water Column [eDNA]
	Count	Mean	SE	RSE	Detects	Count	Mean	SE	RSE	Detects	
311	3	0	0	-	0	3	0	0	-	0	-
313	3	19	18.8	0.99	1	3	0	0	-	0	-
316	3	1025	463.4	0.45	3	3	1	0.6	0.60	1	1846
317	3	140	107.8	0.77	2	3	2	1.5	0.75	2	58
321	3	17	17.3	1.02	1	3	0	0	-	0	-
322	3	901	150.3	0.17	3	3	2	0.8	0.40	2	572
326	3	9425	8477	0.90	2	3	49	25.1	0.51	3	194
331	3	150	63.8	0.43	3	3	1	0.6	0.60	1	231
332	3	544	98.8	0.18	3	3	4	1.8	0.45	2	154
333	3	711	432	0.61	3	3	27	8.5	0.31	3	26
335	3	564	113	0.20	3	3	13	7.8	0.60	3	44
Kansas River	3	418	208	0.50	3	3	8	0.8	0.10	3	79
Wabash River	3	14544	6829	0.47	3	3	185	43.4	0.23	3	8
Wakarusa River	2	1578	1348	0.85	2	3	51	15.9	0.31	3	198

[eDNA] = concentration of target (i.e., Hypophthalmychthys spp.) environmental DNA
Count = the number of samples collected, extracted, and amplified for eDNA
SE = standard error of the mean
RSE = relative standard error of the mean; SE / Mean expressed as a proportion
Detects = the number of positive amplifications out of the count

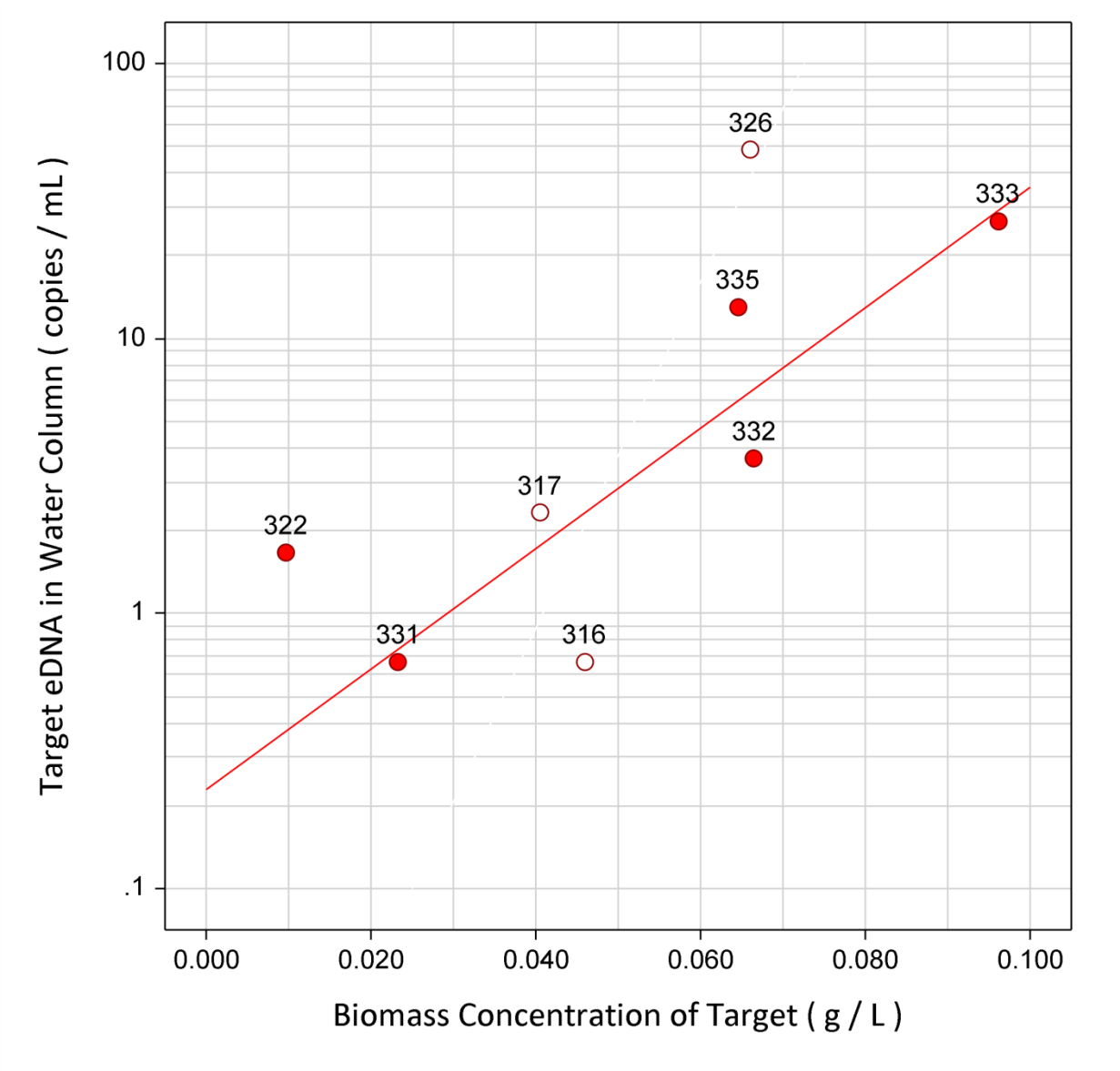


Figure 11. Water column eDNA concentration versus final biomass density of bigheaded carps (Hypophthalmichthys spp.).
Labels indicate experimental pond number. Filled circles indicate a ratio of grass carp biomass to *Hypophthalmichthys* spp. biomass greater than 1, while open circles indicate a ratio less than one. Overall fit line is significant ($p = 0.029$) with an R^2 of 0.58. Individual fit lines for groups based on the ratio of grass carp biomass to bigheaded carp biomass were not significant.

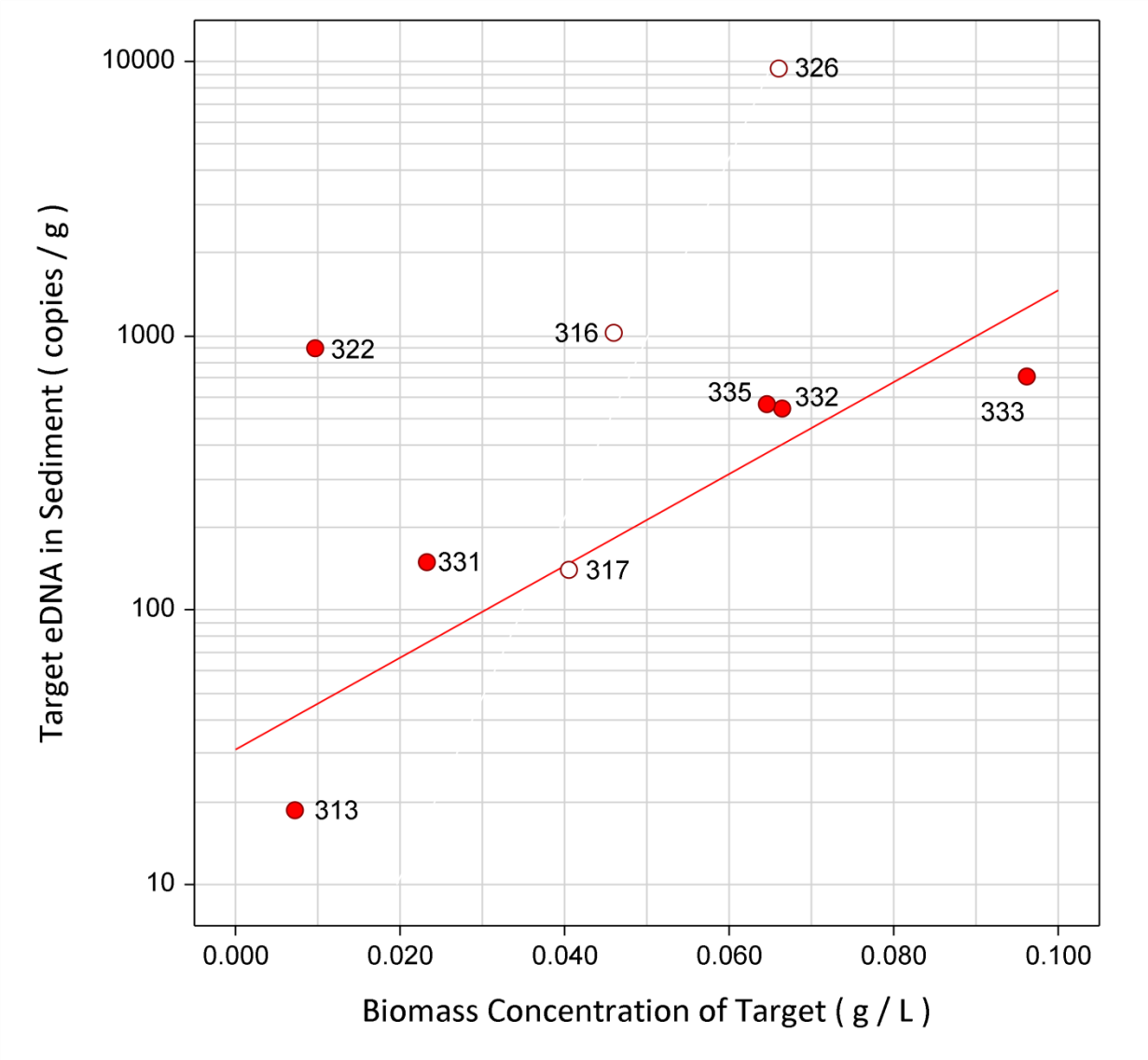


Figure 12. Sediment eDNA concentration versus final biomass of bigheaded carps. Labels indicate experimental pond number.
Labels indicate experimental pond number. Filled circles indicate a ratio of grass carp biomass to *Hypophthalmichthys* spp. biomass greater than 1, while open circles indicate a ratio less than one. The overall fit line for all ponds is significant ($p = 0.035$) with an R^2 of 0.45.

A significant exponential relationship between target eDNA concentration and biomass density (g / L) of the target organism was observed for the experimental ponds in both water column (Figure 11) and sediment (Figure 12) samples, with more variation in water column eDNA concentration ($R^2 = 0.58$) explained by biomass density than in sediment eDNA concentration ($R^2 = 0.45$).

Because the first time step of the degradation study coincided with characterization, it was possible to examine the variation of the target eDNA concentrations in sediment within and between transects. The mean, standard error, and interquartile range of concentrations of bigheaded carp eDNA in individual sediment cores were generally lower than those of composited sediment samples (Table 6). However, relative standard errors were generally lower for composite samples.

Water temperature, pH, dissolved oxygen, five day biological oxygen demand (BOD5), turbidity, suspended chlorophyll *a* concentrations, and specific conductivity were also measured for each pond (see Supplemental Information). Pond water quality measurements were generally similar, but BOD5, turbidity, and suspended chlorophyll *a* in ponds 313 and 332 were significantly higher form other ponds.

Table 6. Variation in eDNA concentration of individual core samples versus transect composite samples by pond.

Pond	Individual Core Sample [eDNA] (copies/g) (Within Transect Variance)					Composite Sample [eDNA] (copies/g) (Between Transect)				
	Count	Mean	SE	IQR	RSE	Count	Mean	SE	IQR	RSE
311	3	0	0	0	-	3	0	0	0	-
313	3	18.7	18.7	56	1.00	1	2000		0	0.00
317	3	140	108	352	0.77	3	7083	1014	3500	0.14
326	3	9425	8477	26342	0.90	2	2875	875	1750	0.30
331	3	150	63.8	213	0.43	3	6250	1323	4500	0.21
332	3	543	98.8	306	0.18	3	45250	31584	105250	0.70
333	3	711	432	1489	0.61	3	10750	2554	8750	0.24

[eDNA] indicates concentration of bigheaded carp eDNA in copies per g.
Mean is the mean concentration of 3 samples, each qPCR amplified in sextuplicate. Non-detects were replaced with zeroes for calculation.
SE is the standard error of the mean.
IQR is the interquartile range of the 18 qPCR amplifications.
RSE is the relative standard error of the mean, SE / Mean

Degradation Study Results

Three composite sediment samples were taken from each of 6 ponds, the samples were fixed at seven time points ranging from 0 to 120 days, and *Hypophthalmichthys* DNA was extracted and amplified using the assay described previously. Initial sediment concentrations of target eDNA ranged from 0 copies/g in the no fish control pond and 93 copies/g in pond 313 to 6485 copies/g in pond 332, then declined over time in all ponds (Figure 13, Table 7). After 2 days bigheaded carp eDNA was no longer recoverable from Pond 313 sediments, and after 10 days bigheaded carp eDNA was no longer recoverable from Pond 326 sediments. These two ponds had the lowest initial sediment eDNA concentrations. The remaining four ponds (316, 331, 332, 333) had measureable target eDNA in the sediment samples through 10 days. Bigheaded carp eDNA was still measurable in two ponds (331, 333) at the end of the study, 120 days after collection.

Sediment degradation was modeled using a simple power law decay model (All Ponds - Table 8; Low Grass Carp ponds - Figure 14; High Grass Carp ponds - Figure 15, Figure 16). Both concentration values and experimental days were log transformed to allow for the following linear model:

$$\log_{10}[eDNA]_{sediment} = A + B\log_{10}(experimental\ days + 1)$$

where A and B are the intercept and slope of the model and one is added to experimental days to allow log transformation of the initial sample points. By subtracting one from the experimental days + 1 and back transforming, the relationship between sediment eDNA and time is exponential:

$$\log_{10}[eDNA]_{sediment} = A + B\log_{10}(experimental\ days + 1 - 1)$$

$$[eDNA]_{sediment} = 10^{(A + B\ \log_{10}(experimental\ days))}$$

$$[eDNA]_{sediment} = 10^{A} * 10^{B\ \log_{10}(experimental\ days)}$$

$$[eDNA]_{sediment} = 10^{A} * (10^{\log_{10}(experimental\ days)})^{B}$$

$$[eDNA]_{sediment} = 10^{A}\ experimental\ days^{B}$$

where $[eDNA]_{sediment}$ is the sediment concentration of target eDNA in copies/g, A is the intercept of the linear model, which corresponds to the initial amount of target eDNA present before degradation began. B is the slope of the linear model, which corresponds to the rate at which the initial eDNA concentration declines over time. If B is zero, that is there is no slope or in other words no decay, then the eDNA concentration is equal to the initial concentration. Experimental days refers to the number of days elapsed from initial sampling until preservation of the sediment sample.

For a parameter estimate to be significantly different from zero, the confidence interval of the estimate must not include zero. Based on the 95% confidence intervals of the slope estimates, four ponds had regression slopes that were significantly different from zero (Figure 17, Table 8). Of these four ponds, there were two pairs of overlapping estimates, with one pair (326, 332) having significantly steeper regression slopes than the other (331, 332).

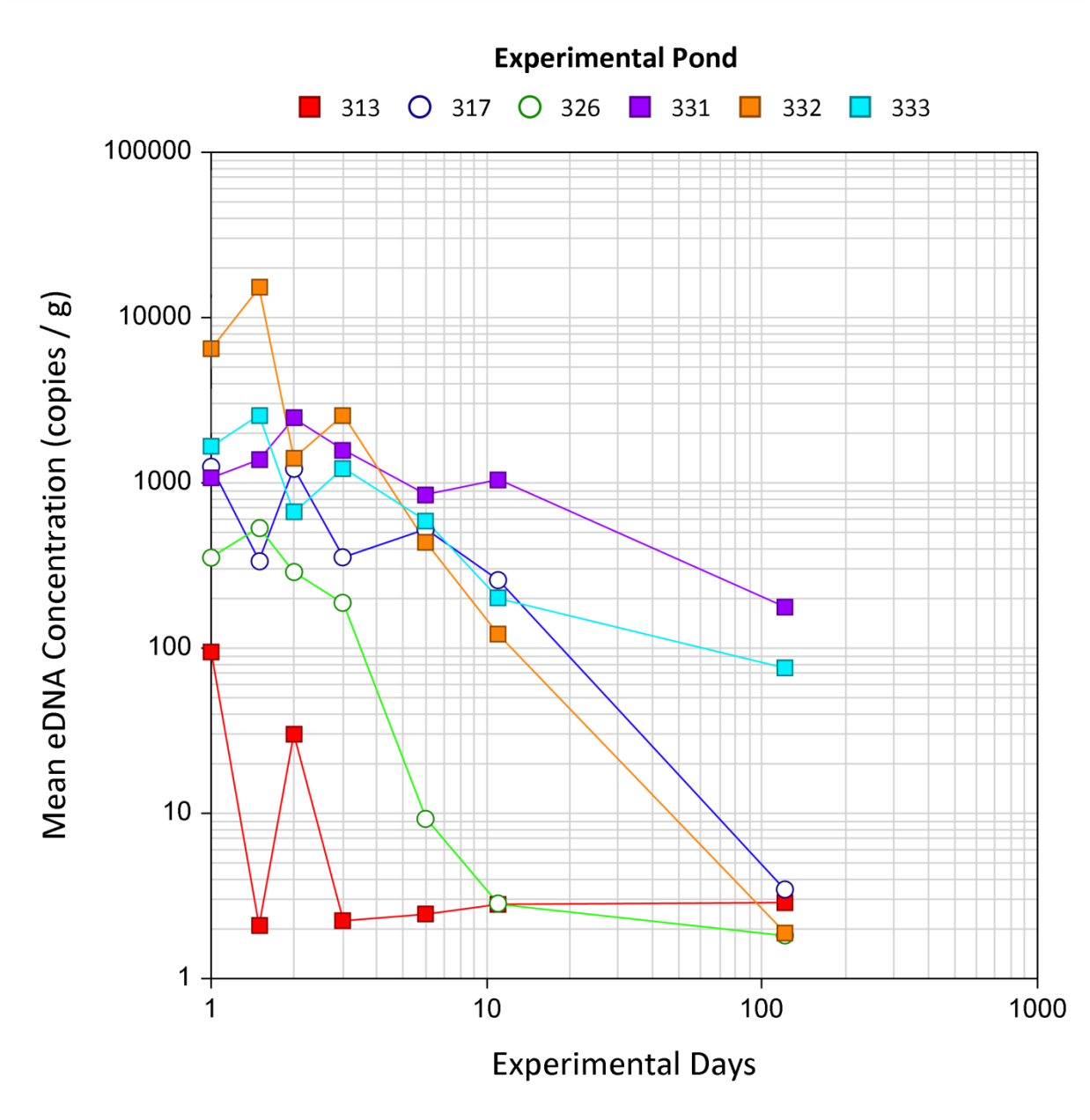


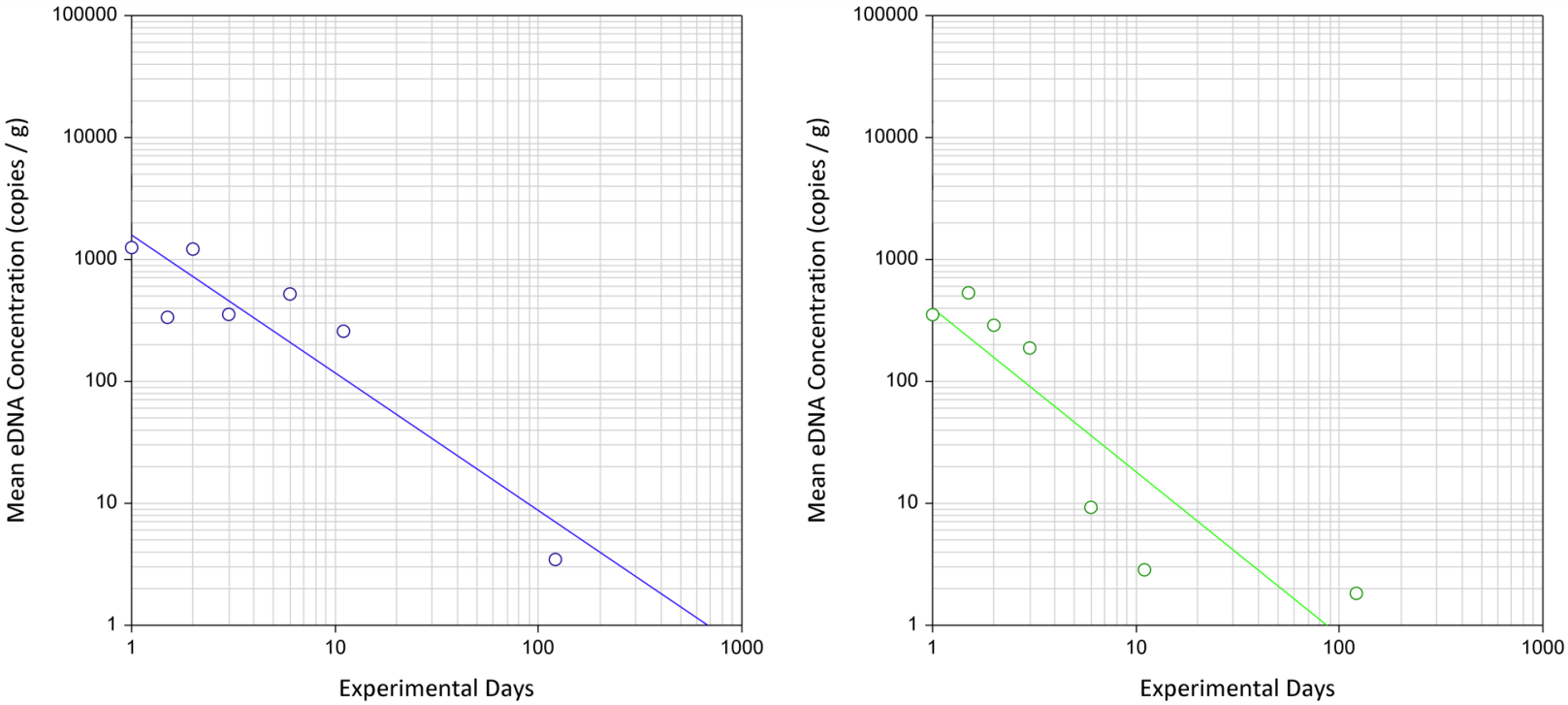
Figure 13. Sediment eDNA concentrations over time by pond.
Values represent the mean of 3 composite samples, each qPCR amplified in sextuplicate. Technical replicates that did not amplify were replaced with zeros for purposes of mean calculations. Experimental days were transformed by adding 1 to each value in order to display initial (i.e., experimental day zero) values on a logarithmic scale. Filled squares and open circles designate ponds with high and low grass carp to bigheaded carp biomass, respectively.

Table 7. Sediment eDNA concentration by pond and days until preservation.
Mean represents the mean value of 3 replicate samples per pond. Green shading indicates ponds with grass carp to bigheaded carp biomass ratio greater than one.

Days Until Preservation	Pond	Sediment [eDNA] (copies / g)							
		Mean	SD	SE	Min	Max	Range	IQR	RSE
0	311	0	0	0	0	0	0	0	-
	313	93.3	162	93.3	0	280	280	280	1.00
	317	1251	134	77.4	1097	1339	242	242	0.06
	326	352	413	239	0	807	807	807	0.68
	331	1071	434	251	723	1558	835	835	0.23
	332	6485	7917	4571	199	15377	15178	15178	0.70
	333	1664	492	284	1096	1958	862	862	0.17
0.5	313	0	0	0	0	0	0	0	-
	317	335	260	150	41.7	539	497	497	0.45
	326	532	662	382	80.5	1292	1211	1211	0.72
	331	1381	234	135	1222	1650	428	428	0.10
	332	15267	23873	13783	0	42778	42778	42778	0.90
	333	2551	2228	1287	140	4534	4395	4395	0.50
1	313	28.5	49.4	28.5	0	85.6	85.6	85.6	1.00
	317	1216	677	391	566	1918	1351	1351	0.32
	326	289	51.1	29.5	254	347	93.2	93.2	0.10
	331	2480	1526	881	757	3663	2906	2906	0.36
	332	1409	1756	1014	350	3436	3085	3085	0.72
	333	668	103	59.3	577	779	202	202	0.09
2	313	0	0	0	0	0	0	0	-
	317	354	225	130	137	586	449	449	0.37
	326	186	322	186	0	558	558	558	1.00
	331	1570	882	510	788	2527	1739	1739	0.32
	332	2549	2896	1672	0	5698	5698	5698	0.66
	333	1219	668	385	817	1990	1173	1173	0.32

Days Until Preservation	Pond	Sediment [eDNA] (copies / g)							
		Mean	SD	SE	Min	Max	Range	IQR	RSE
5	313	0	0	0	0	0	0	0	-
	317	521	219	126	290	726	436	436	0.24
	326	7.63	13.2	7.63	0	22.9	22.9	23	1.00
	331	844	613	354	301	1508	1207	1207	0.42
	332	436	478	276	0	948	948	948	0.63
	333	587	540	312	260	1210	950	950	0.53
10	313	0	0	0	0	0	0	0	-
	317	257	224	129	0	412	412	412	0.50
	326	0	0	0	0	0	0	0	-
	331	1041	625	361	606	1756	1150	1150	0.35
	332	120	208	120	0	359	359	359	1.00
	333	200	346	200	0	600	600	600	1.00
120	313	0	0	0	0	0	0	0	-
	317	0	0	0	0	0	0	0	-
	326	0	0	0	0	0	0	0	-
	331	176	182	105	0	364	364	364	0.60-
	332	0	0	0	0	0	0	0	-
	333	74.3	129	74.3	0	223	223	223	1.00

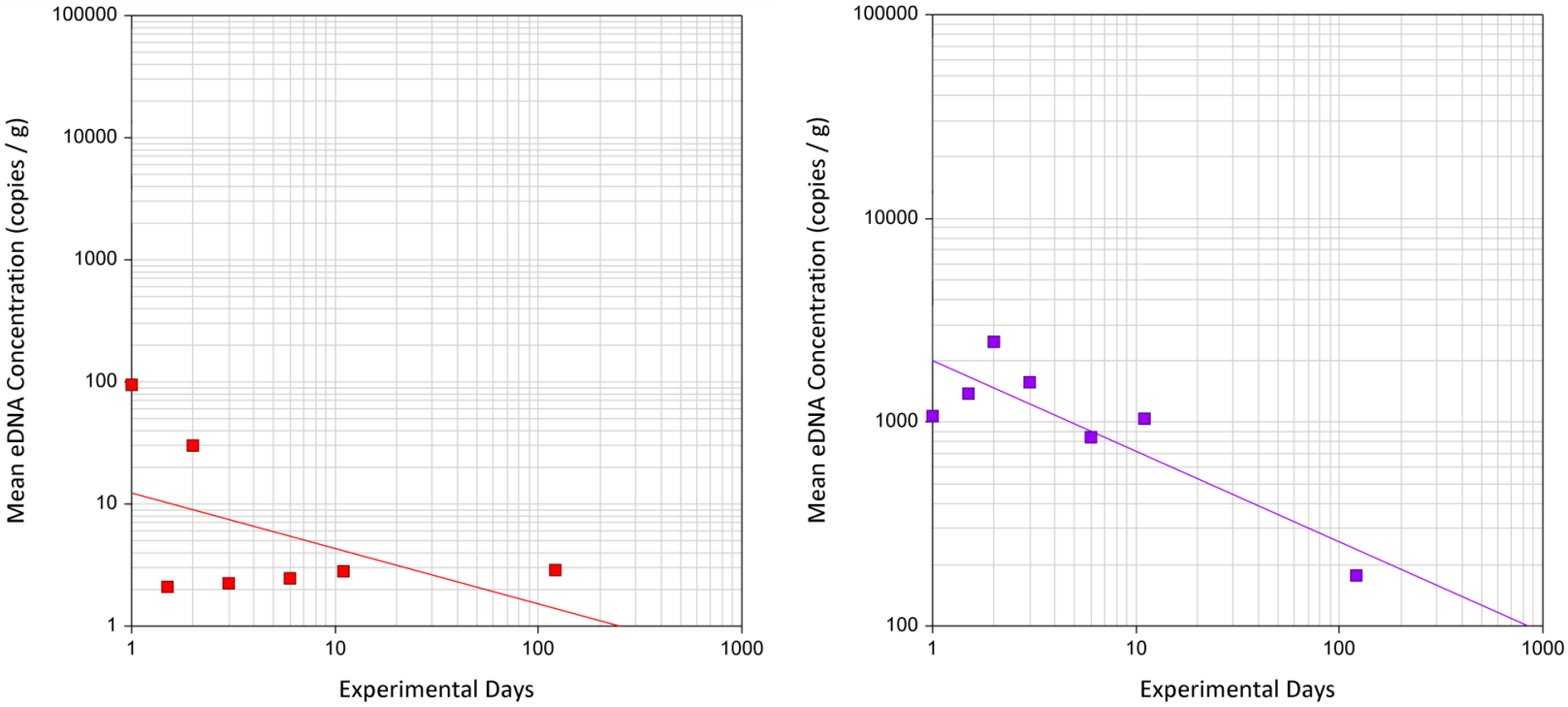
Mean is the average of 3 samples, each amplified in sextuplicate
SD is the standard deviation of the mean
SE is the standard error of the mean
Min, Max, and Range are the minimum value, maximum value, and (Max – Min), respectively.
IQR is the interquartile range (i.e., the 75^{th} percentile value minus the 25^{th} percentile value).
RSE is the relative standard error, SE / Mean



(a) Pond 317

(b) Pond 326

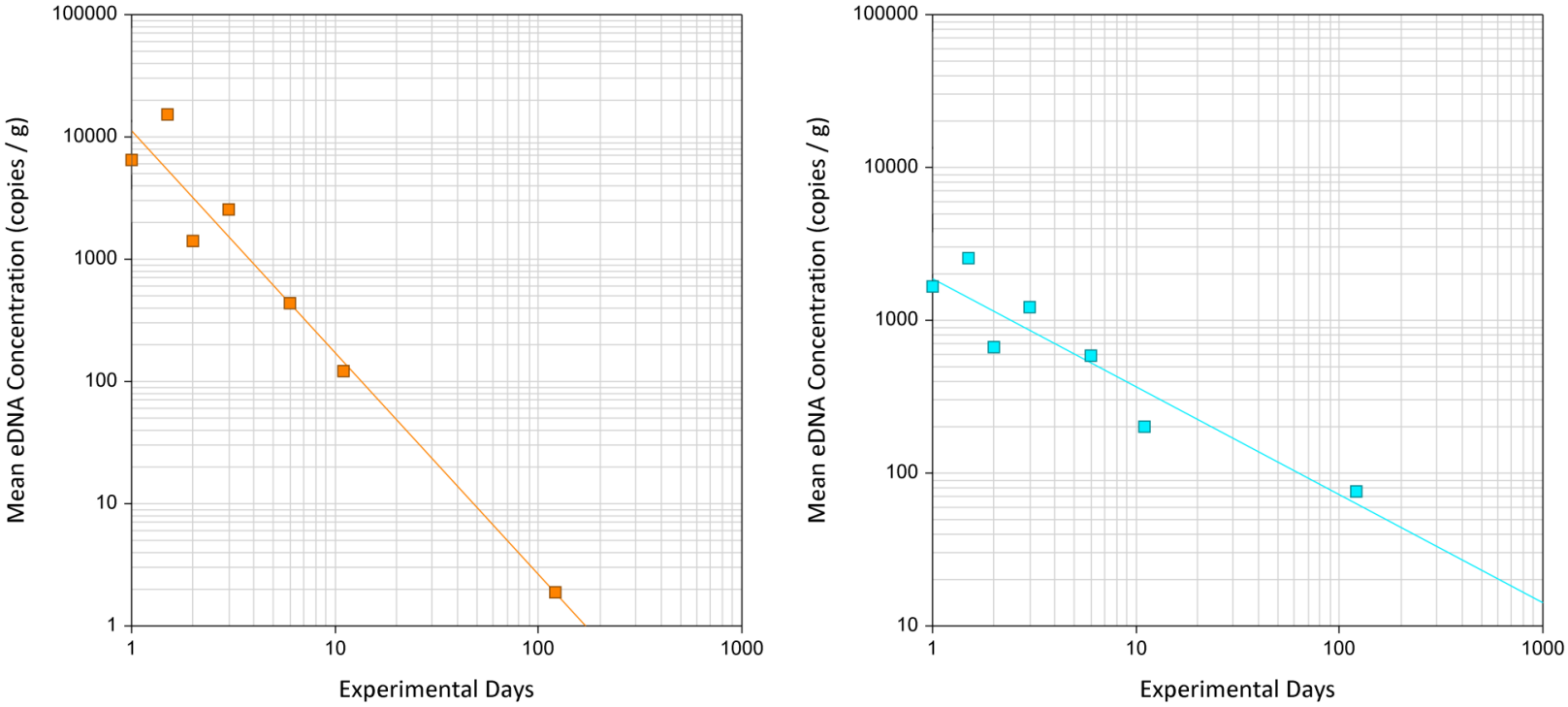
Figure 14. Scatterplots of sediment eDNA concentration over time for ponds with a grass carp to bigheaded carp biomass ratio less than 1 (a) Pond 317 and (b) Pond 326.
Plotted concentrations represent mean values of 3 replicate samples, each of which was qPCR amplified in sextuplicate. Trend lines represent estimated degradation models. See Table 8 for regression parameters.



(a) Pond 313

(b) Pond 331

Figure 15. Scatterplots of sediment eDNA concentration over time for ponds with grass carp to bigheaded carp biomass ratios greater than 1 (a) Pond 313 and (b) Pond 331.
Plotted concentrations represent mean values of 3 replicate samples, each of which was qPCR amplified in sextuplicate. Trend lines represent estimated degradation models. See Table 8 for regression parameters.



(a) Pond 332

(b) Pond 333

Figure 16. Scatterplots of sediment eDNA concentration over time for ponds with grass carp to bigheaded carp biomass ratios greater than 1 (a) Pond 332 and (b) Pond 333.
Plotted concentrations represent mean values of 3 replicate samples, each of which was qPCR amplified in sextuplicate. Trend lines represent estimated degradation models. See Table 8 for regression parameters.

Table 8. Estimates of sediment degradation rate model parameters by pond.
Models are Y = AX + B, where Y = log (concentration of target eDNA in sediment), A = Slope, X = log (experimental days), and B = Intercept. Mean is the mean of 3 composite samples per pond, whose values were determined by averaging sextuplicate qPCR amplifications. Lower 95% CL and Upper 95% CL are the lower and upper confidence limits of the estimate. By definition, this model assumes a power law relationship between sediment eDNA concentrations and time. Parameter units are log(copies/g) for the Intercept and log(copies/g) per log(days) for the slope. See text for further elaboration.

Parameter	Statistic	Pond					
		313	**317**	**326**	**331**	**332**	**333**
Intercept	Mean	1.35	2.95	2.98	3.30	4.05	3.28
	Lower 95% CL	0.18	2.47	2.34	3.02	3.63	2.99
	Upper 95% CL	2.52	3.43	3.61	3.58	4.47	3.57
Slope	Mean	-1.13	-0.48	-2.31	-0.44	-1.81	-0.71
	Lower 95% CL	-3.14	-1.30	-3.40	-0.74	-2.26	-1.01
	Upper 95% CL	0.88	0.35	-1.23	-0.15	-1.37	-0.40
R^2		0.38	0.39	0.90	0.76	0.96	0.88
p-value		0.19	0.18	0.004	0.011	0.0001	0.002

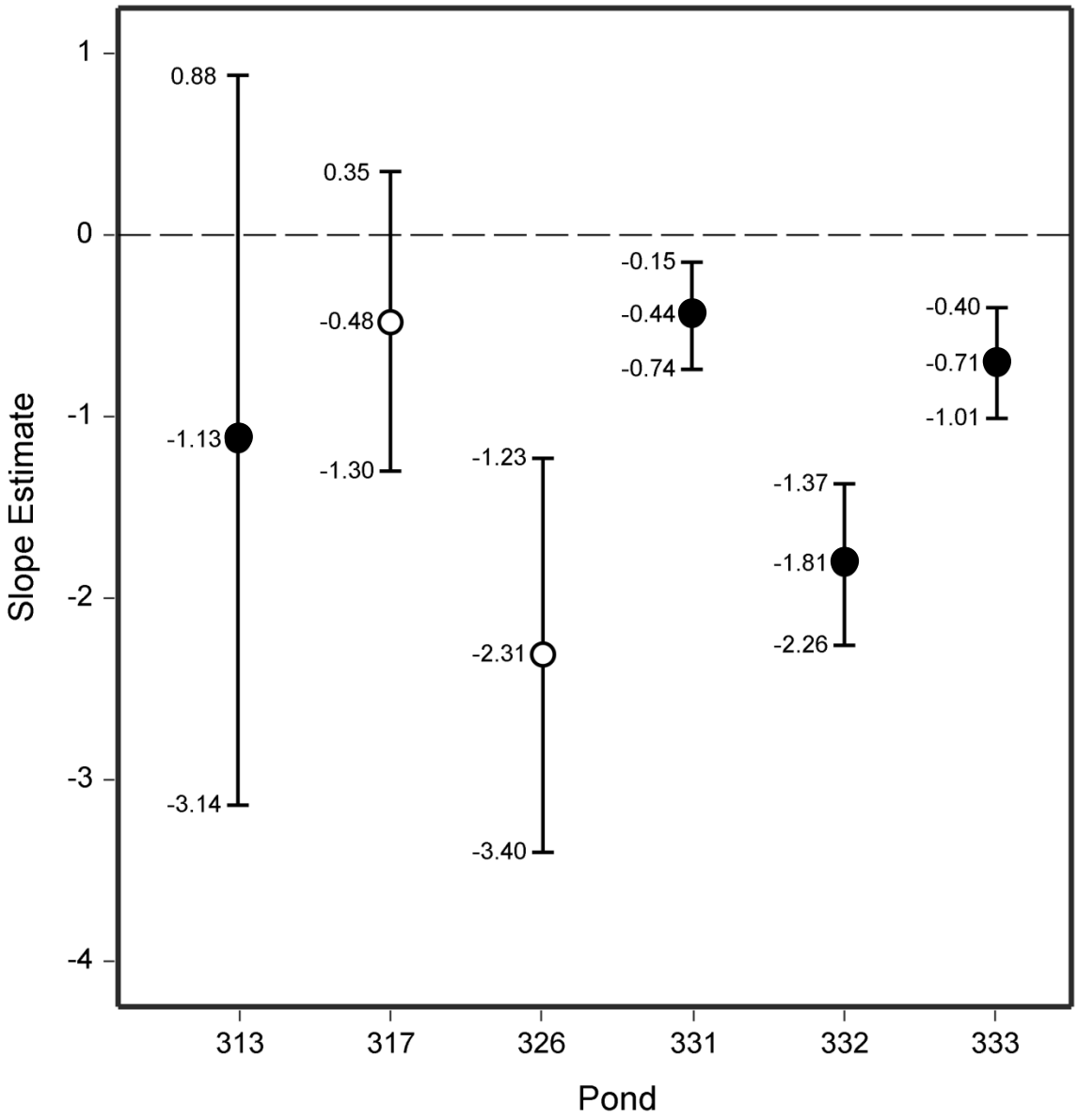


Figure 17. Estimates of the slope of sediment eDNA degradation.
Estimates are based on regressions as described in the text. Circles indicate mean estimates, and bars indicate the upper and lower 95% confidence interval. Open and filled circles indicate ponds with grass carp to bigheaded carp biomass ratios less than and greater than 1, respectively. Medians and upper and lower bounds are as shown. Where the bars do not overlap, there is 95% confidence that the true means of the slopes are different. Similarly, where bars overlap the dashed line, the 95% confidence band includes zero.

DISCUSSION

In designing the experiment we made several predictions about both the characterization and degradation of bigheaded carp eDNA concentrations in the experimental units of this study. Specifically, in terms of characterization we predicted that: (1) sediments would have higher bigheaded carp eDNA concentrations than the overlying water column; (2) higher resuspension would yield higher water column concentrations of bigheaded carp eDNA; and (3) higher amounts of the target fish would yield both higher sediment concentrations and higher water column concentrations of bigheaded carp eDNA. We also predicted that higher initial concentrations of bigheaded carp eDNA would yield higher degradation rates, but that higher resuspension would yield lower degradation rates.

Based on previous evidence of increased extracellular DNA concentration in marine sediments (Dell'Anno et al. 2002, Corinaldesi et al. 2005, Corinaldesi et al. 2008, Corinaldesi et al. 2011), the size, settling velocity, and nature of fish wastes and other associated particles (Sarà et al. 2004, Muhammad 2012, Saba and Steinberg 2012) and recent observations of the particle size and distribution of eDNA bearing material associated with fishes (Turner et al. 2014), we had predicted that sediment concentrations of *Hypophthalmichthys* eDNA would be higher than water column concentrations. Essentially, the majority of bigheaded carp eDNA is associated with relatively large particles that are large enough to sink rapidly, thereby transporting them from the water column to surficial sediments. Evidence from our sediment characterization study supports this hypothesis, with target eDNA concentrations 8 to 1800 times higher in sediments than in the water column, assuming a 1 g to 1 mL equivalency. This finding was consistent across all 10 experimental ponds and all 3 natural river sites included in this study (Figure 10, Table 5). Relatively higher concentrations of bigheaded carp eDNA were found in sediments than in the water column regardless of biomass density of the target fish, and sediment concentrations of bigheaded carp eDNA were correlated with water column concentrations

(Figure 11, Figure 12). This finding and its implications for biological conservation are discussed at length in my co-authored paper (Turner et al. 2015).

Sediment concentrations of bigheaded carp eDNA were also found to be relatively lower and less variable between cores within the same transect than between transect composite samples (Table 6). In other words, it appears that 3 single cores are less likely to collect high copy number samples than 3 composite samples of 3 cores each. It is highly unlikely that eDNA is uniformly distributed in sediments, and rather more likely to be clumped. Fewer samples appear to collect fewer high copy number clumps, yielding relatively lower concentrations of target eDNA with relatively lower variation. However, the relative standard errors (RSEs) were generally lower with composite samples. Pilliod et al. (2013) suggested that samples with lower relative standard errors are likely more representative estimates of heterogeneous eDNA distributions in aquatic systems. Therefore, transect composite values may be more reliable estimates of the representative target eDNA concentration in sediments than separate cores.

We also predicted that an increased amount of the target fish would be associated with increased concentrations of the target fish eDNA in both water column and sediment samples. This hypothesis was supported by statistically significant relationships between increasing bigheaded carp biomass density and increasing target eDNA concentrations, both in the water column (Figure 11) and in sediments (Figure 12). About half of the variation in eDNA concentration was explained by bigheaded carp biomass density. Similar positive relationships between the number or density of macroorganisms and environmental concentrations of their DNA were found in bullfrogs (Ficetola et al. 2008, Dejean et al. 2011), endangered amphibians (Goldberg et al. 2011, Thomsen et al. 2012), and other fish (Takahara et al. 2012, Evans et al. 2015).

Resuspension may affect both water column and sediment concentrations of bigheaded carp eDNA as materials move back and forth between the water column and surficial sediment compartments. Ponds with higher resuspension were predicted to yield higher water column concentrations of bigheaded

carp eDNA. Clay particles comprise over one third of the mineral substrate in the experimental ponds at KUFS. Clay particles are known to bind DNA and enzymes (Demaneche et al. 2001, Theng 2012) and to resuspend both on their own and in combination with bound DNA and DNA bearing partilces (Avnimelech et al. 1999, Jamieson et al. 2005, Vanoni 2006, Roozen et al. 2007).

Bigheaded carp eDNA concentrations appear to have also been affected by changes in environmental conditions created by grass carp. For ponds with relatively high grass carp densities (i.e., ponds with a ratio of grass carp biomass to bigheaded carp biomass of one or greater), biomass concentrations of bigheaded carp were generally higher than those in ponds with relatively low grass carp densities, even though there were fewer individuals present (Table 4). For example, pond 335 had five bigheaded carp, but it had a higher water column eDNA concentration than both ponds 316 and 317, each with 13 bigheaded carp.

Overall, differences in sediment eDNA concentration appear to correlate with increased bighead carp eDNA concentration. Ponds 333, 335, and 332 all have higher bigheaded carp biomass density and higher bigheaded carp eDNA concentrations than ponds 317 and 316. Increased biomass in the high density grass carp ponds may be related to higher concentrations of available nutrients released during consumption and removal of macrophytes. Since bigheaded carps are filter-feeding primary consumers, a majority of their nutrition comes from filtration of algae (Kolar et al. 2005). Observations of algal blooms in high density grass carp ponds (Figure 4a), coupled with significantly higher suspended chlorophyll a concentrations (Figure 8), both suggest that increased food supply was available to bigheaded carp in these ponds. Increased bigheaded carp biomass is a direct result of larger individuals, and elevated growth is likely a result of increased food supply. Increased biomass alone, however, does not completely explain the elevated eDNA concentrations. For example, pond 331 had a similar water column eDNA concentration to both ponds 316 and 317, even though 316 and 317 had more than four times the individuals and approximately twice the biomass of bigheaded carps (Table 4, Table 5, Figure 18). Also, pond 326 had both the highest water column concentration of bigheaded carp eDNA and the

highest number of individuals, but its biomass concentration was similar to both ponds 332 and 335 and less than pond 333 (Table 4, Table 5, Figure 18). For both groups of ponds, the relationship of bigheaded carp water column eDNA was largely explained by the ratio of grass carp biomass to bigheaded carp biomass (Figure 18, Table 9). A similar trend was observed in sediment concentrations of bigheaded carp eDNA (Figure 19, Table 9).

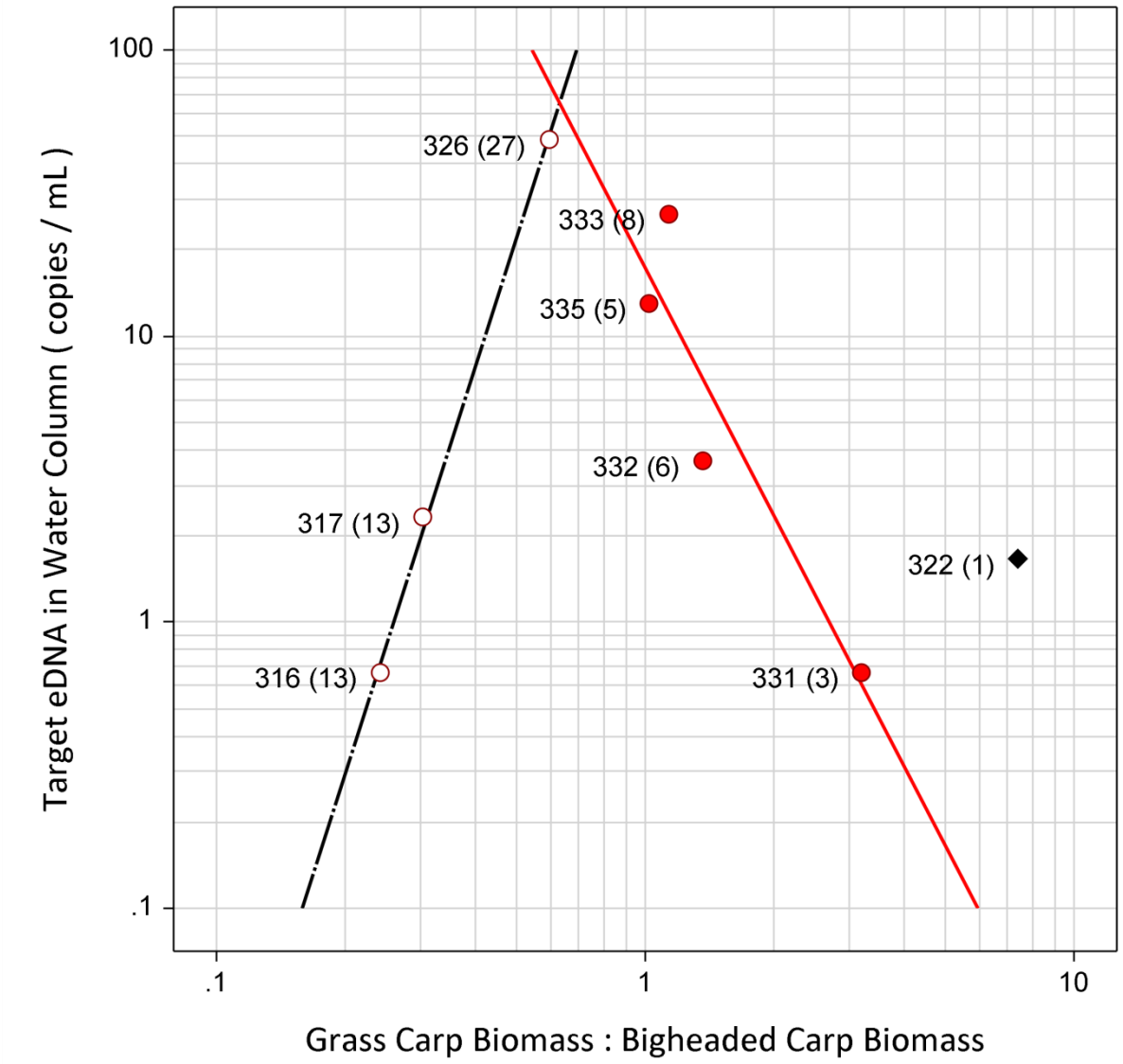


Figure 18. Water column concentration of *Hypophthalmichthys* eDNA by biomass ratio of grass carp to bigheaded carps.
Open and filled symbols respectively represent fewer or more grass carp present in the pond (i.e., biomass ratio less than or greater than one). Notations indicate the pond number with the number of bigheadded carp individuals in parenthesis. Broken and solid lines represent trends for the groups with biomass ratio less than one and greater than one, respectively ($R^2 = 0.998$, $p = 0.028$; $R^2 = 0.856$, $p = 0.0749$). Pond 322 (filled diamond) had only 1 bigheaded carp, an order of magnitude lower bighead carp biomass, and the highest grass carp to bigheaded carp biomass in the study. It was therefore treated as an outlier and excluded from the regression. Pond 313 had no amplification from water samples.

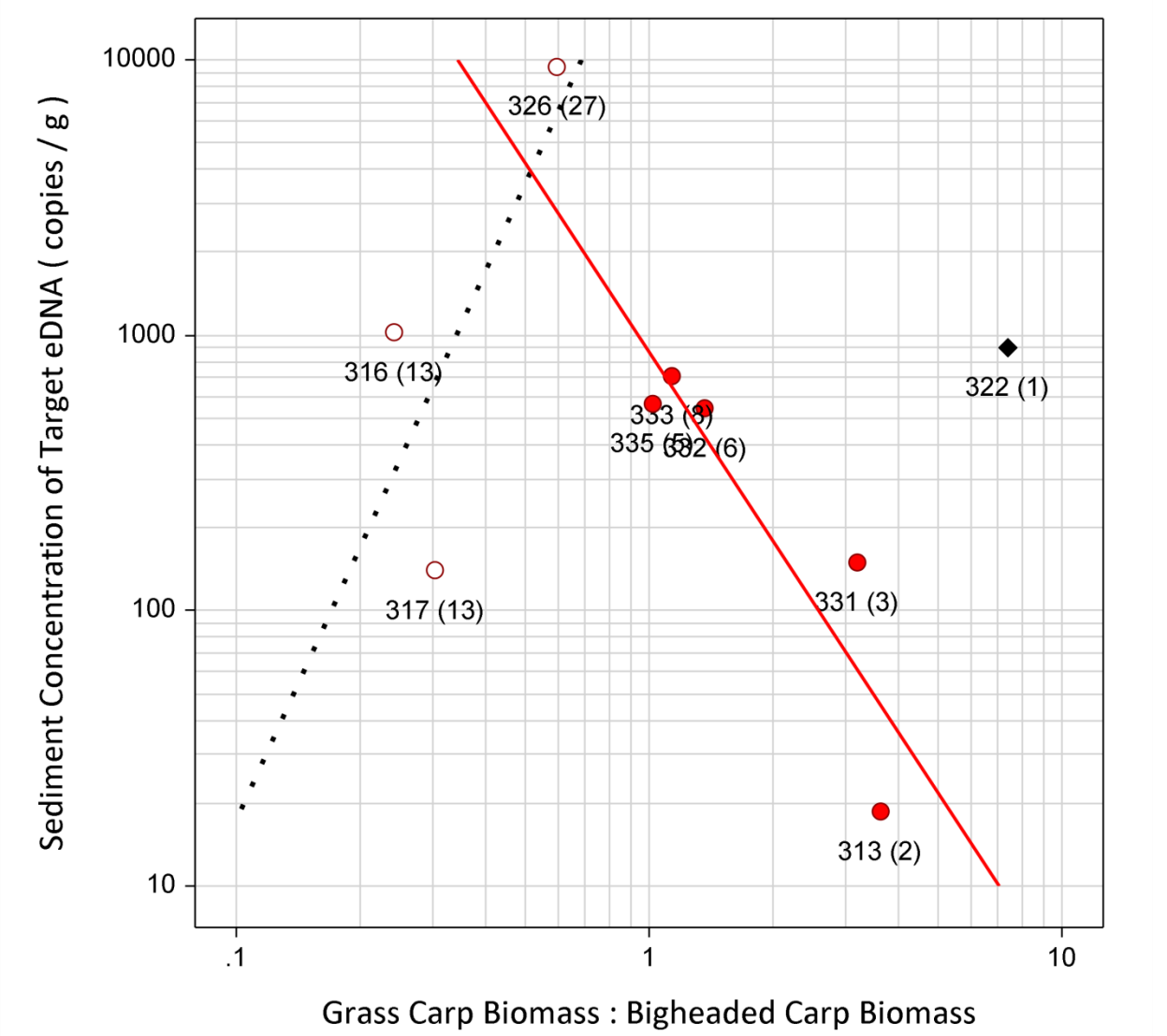


Figure 19. Water column concentration of *Hypophthalmichthys* eDNA by biomass ratio of grass carp to bigheaded carps.
Open and filled symbols respectively represent fewer or more grass carp present in the pond (i.e., biomass ratio less than or greater than one). Notations indicate the pond number with the number of bigheadded carp individuals in parenthesis. Broken and solid lines represent trends for the groups with biomass ratio less than one and greater than one, respectively ($r^2 = 0.549$, $p = 0.0.469$; $r^2 = 0.856$, $p = 0.0749$). Pond 322 (filled diamond) had only 1 bigheaded carp, an order of magnitude lower bighead carp biomass, and the highest grass carp to bigheaded carp biomass in the study. It was therefore treated as an outlier and excluded from the regression.

Table 9. Regression parameters for eDNA concentration versus the ratio of grass carp biomass to bigheaded carp biomass.
Regression model is logY = A + B logX, where Y is the mean eDNA concentration, X is the ratio of grass carp biomass to bigheaded carp biomass, and A and B are the intercept and slope, respectively.

Environmental Compartment	GC to BC Biomass Ratio	Model Parameters			
		Intercept	Slope	R^2	p-value
Water Coumn	< 1	2.744	4.668	0.998	0.0283
	1 or greater	1.239	-2.886	0.856	0.0749
Sediment	< 1	4.542	3.303	0.549	0.469
	1 or greater	2.934	-2.286	0.806	0.0386

GC to BC Biomass Ratio is the ratio of grass carp biomass to bigheaded carp biomass

A threshold appears to occur when biomass concentrations of grass carp and bigheaded carp are approximately equal (i.e., the biomass ratio of grass carp to bigheaded carp is near 1). Below this value, bioturbation and ecological alteration by grass carp were less prevalent, whereas they were readily observable in ponds with ratios greater than one. Several implications for monitoring arise from this observation. First, the alteration of habitats that promote algal growth appears to enhance growth of bigheaded carp as well, as bigheaded carp in high grass carp ponds have significantly higher average biomass than those in low grass carp ponds (Table 4; $p = 0.020$, Kruskall-Wallis test). If fish are present in two ponds with equal numbers, but one pond has more biomass, then measurable eDNA concentrations should be higher in the pond with more biomass of target fish, based on biomass trends found in this study. Second, the increased mixing from grass carp may resuspend eDNA that would have settled to the bottom, allowing for both additional contact with suspended clay particles and additional contact with enzymes in the water column. In systems with significant clay concentrations in the sediment, increased contact may lead to increased attachment of eDNA. Such attachment may provide protection from enzymatic hydrolysis, prolonging the signal of eDNA in well-mixed or high grass carp systems as compared to non-mixed or low grass carp systems. Third, consumption of macrophytes by grass carp is known to mobilize nutrients and increase fecal and bacterial loading (Lembi et al. 1978, Dibble and

Kovalenko 2009). Enzymatic hydrolysis is likely the most important mode of degradation in natural aquatic systems (Barnes et al. 2014), and production of enzymes tends to increase with increases in metabolism in sediments (Dell'Anno and Corinaldesi 2004, Corinaldesi et al. 2008, Corinaldesi et al. 2011, Zinger et al. 2012). Therefore, degradation rates may be higher in high grass carp systems due to increased enzyme concentrations. The interplay between increased growth, increased attachment, and increased enzyme concentrations creates a complicated feedback network that makes prediction and modeling of in situ eDNA concentrations challenging. Based on observations from this study, the highest concentrations of eDNA seem to occur when grass carp biomass is slightly smaller than bigheaded carp biomass (i.e., biomass ratio slightly less than one), or when environmental conditions promote growth, mix moderately, and limit hydrolytic enzyme production.

Degradation of bigheaded carp eDNA in sediments from six ponds was observed over 120 days. Four of the six ponds still had measurable concentrations at 120 days (Figure 13, Table 7). These findings corroborate detection of bigheaded carp eDNA in sediment samples from pond 321, where the only stocked bigheaded carp had been removed 132 days prior to sampling (Table 5). Recovery from environmental samples at these time scales suggests that fish eDNA may persist significantly longer in aquatic environments than the hours to days that had been previously reported (e.g., Dejean et al. 2011, Thomsen et al. 2012).

For each of the six ponds in the degradation study, the decay model

$$[\text{eDNA}]_{sediment} = 10^{A}(\textit{experimental days} + 1)^{B}$$

was fit using a log-log transformation to allow for a simple linear regression to predict the relationship (All Ponds - Table 8; Low Grass Carp ponds - Figure 14; High Grass Carp ponds - Figure 15, Figure 16). Power models have been used to predict decay rates of organic material in marine sediments (Arndt et al. 2013) and recent studies have suggested that enzyme kinetics in complex matrices with variable diffusion rates may be better fit with power law functions than traditional Michaelis-Menten kinetics (Hyojoon and

Kook Joe 2007, Vasilescu et al. 2013). Based on the 95% confidence intervals of the slope estimates, two pairs of ponds had overlapping, non-zero slopes (Figure 17). Because the slope estimate of the model represents a power function, the relative relationship between the actual eDNA concentration of one pond to the other ponds changes as a function of the time elapsed to the power B. By plotting the model predictions as proportions of the initial eDNA concentration, it can be seen that less than 5% of the initial sediment eDNA in ponds 326 and 332 is expected to remain by day 10, whereas approximately 20% and 35% are expected to remain in ponds 333 and 331, respectively (Figure 20).

Based on the concept of substrate-limited enzymatic hydrolysis, it was predicted that higher eDNA concentrations would yield faster degradation rates. Day 0 eDNA concentration (Table 7) and rate of degradation slope estimate (Table 8) were not correlated for the four ponds with non-zero slope estimates ($p = 0.79$) when considered together, but were highly correlated for the three high density grass carp ponds 331, 332, and 333 ($R^2 = -0.99$, $p = 0.055$). This suggests that initial concentration is a significant driver for degradation rates in well-mixed, turbid systems, but may not be in other ecological conditions. For example, in ponds 331, 332, 333, enzymatic hydrolysis may be the dominant mechanism of degradation, but in pond 326 where the water was relatively clearer, dissolved nutrients were likely lower (no algal blooms, macrophyte presence), and microbial activity may have also been lower (lower BOD5). Degradation by ultraviolet light may have had a more important impact in this pond than in the higher density grass carp ponds.

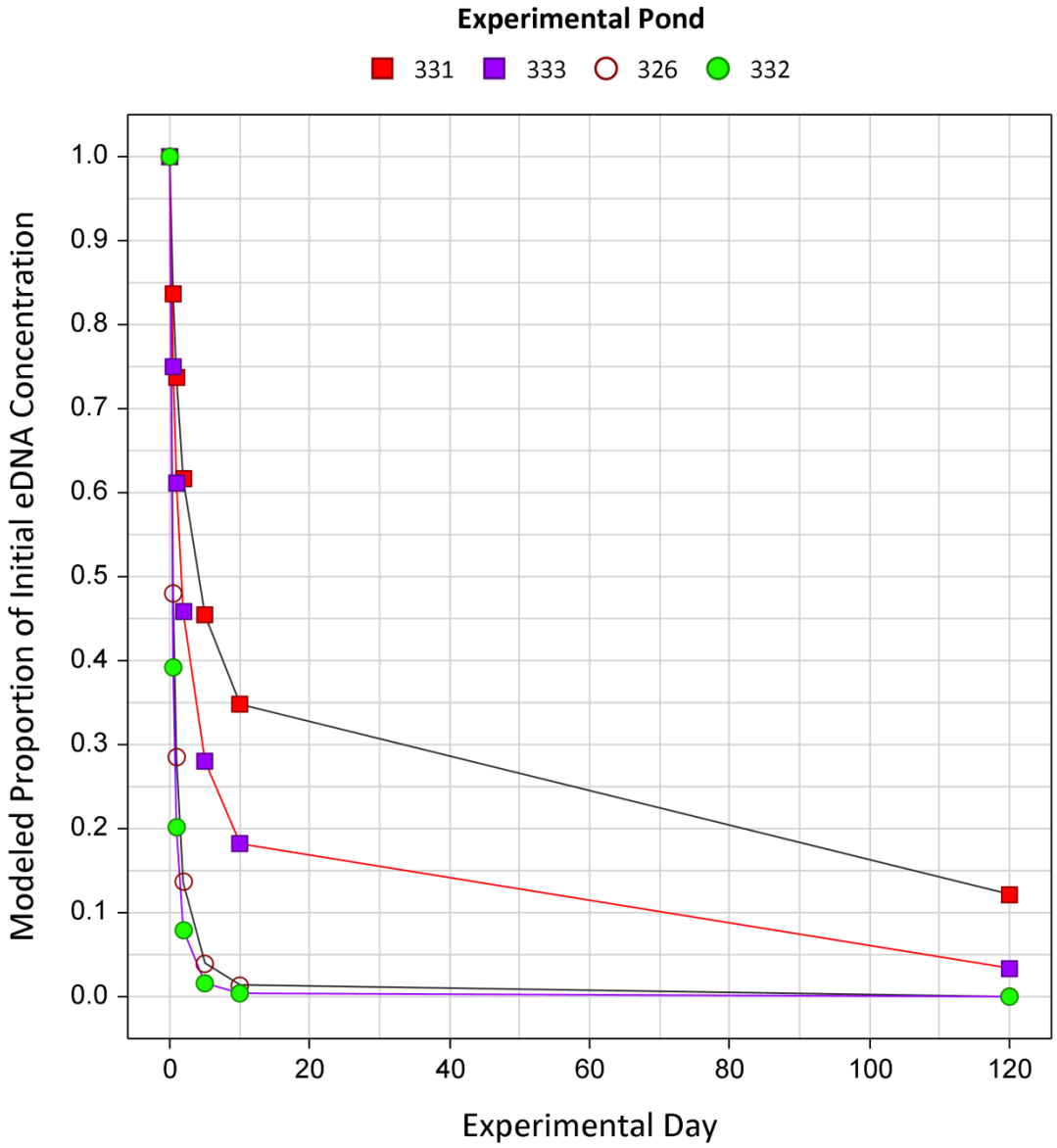


Figure 20. Proportion of initial eDNA concentration remaining by experimental day by pond. Plotted values are the X^B term from the log-log sediment degradation model, $Y = 10^A X^B$, where A is the model intercept, B is the model slope, Y is the eDNA concentration, and X is the number of experimental days plus one. This term is represents the proportional factor by which initial concentrations are reduced, and it varies by pond with the elapsed time. Squares represent one pair of ponds with overlapping slope estimates (B = -0.44 for 331 and B = -0.71 for 333) and circles represent the other pair (B= -2.31 for 326 and B = -1.81 for 332). Filled symbols indicate ponds with high grass carp, while open symbols indicate the pond with low grass carp to bigheaded carp biomass ratio. Additional model parameters appear in Table 8.

Given the observed bioturbation and known association of DNA and DNA bearing particles with clays and organic substrates similar to those in the experimental ponds, it was also hypothesized that

resuspension would lower observed degradation rates. However, no significant trends were observed between degradation slope estimates and the number of grass carp, biomass of grass carp, or the biomass ratio of grass carp to bigheaded carp. Additional studies should consider monitoring ecological conditions such as nutrient concentrations and direct sedimentation and resuspension rates, as well as more direct measures of degradation mechanisms such as hydrolytic enzyme concentrations, ultraviolet light levels, and degradation of *in situ* surrogate DNA. In particular, highly replicated degradation trials under variable light, dark, and enzyme concentrations may provide better insight into the fine-scale mechanisms that directly affect fish eDNA concentration in sediments.

CONCLUSIONS

1) Fish eDNA concentrations are higher in sediment than the overlying water column and are correlated with biomass density of the target fish.

2) Resuspension of sediments and ecological changes associated with grass carp can obscure biomass effects.

3) Internal positive controls can confirm lack of inhibition during qPCR amplification, and are recommended for every assay.

3) Fish eDNA in sediments is detectable and quantifiable even 132 days after removal of the fish.

4) Composite samples of three sediment cores exhibit lower relative standard errors than samples from individual cores.

5) Degradation in sediments can be approximated as $Y = 10^{A}X^{B}$, where Y is the eDNA concentration, X is the number of experimental days plus one, and A and B are the intercept and slope, respectively, of a linear model of log-log transformed data.

6) Enzymatic hydrolysis appears to drive degradation of fish eDNA in ponds, with a more dominant role in ponds with relatively high grass carp populations and a potentially less dominant role in other pond systems.

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CHAPTER 5: Overall conclusions and implications for future research

Environmental DNA shows continuing promise as an indicator for environmental monitoring. eDNA from both large and small vertebrates can be recovered from water and sediment, quantified, and related to biomass of the target organism. This relationship with biomass held within habitats with varying degrees of disturbance from macrophyte removal and bioturbation, also suggesting relative quantification may be possible within similar habitat types. Therefore, eDNA has potential for relative quantification of target organisms if habitat and seasonal effects can be better understood. However, considerable challenges remain for environmental monitoring through quantification of eDNA.

Habitat differences affect eDNA concentrations and degradation rates through space and time. As shown in this study and others (e.g., Pilliod et al. 2013), relative standard errors of eDNA measurements may be high in spite of rigorous field and laboratory protocols, simply by the heterogeneous nature of site occupancy, material deposition, or environmental compartment where the eDNA resides. Additionally, flow dynamics in aquatic systems likely concentrate target eDNA in sediments in areas with quiescent waters, where it can rapidly reduce in concentration, yet persist on the order of months without additional source material. Similarly, in turbulent waters, eDNA and other materials may be resuspended or well-mixed, thereby altering the dynamics of production, shearing, settling, and decay. In lotic networks, hydraulic models for particle transport may provide insight into the pathways and hotspots where target eDNA may travel through specific systems. Therefore, increased understanding of how eDNA compartmentalizes between suspended, settled, and attached phases will be imperative for prediction.

Moreover, the continuum of the state of eDNA in the environment (i.e., eDNA bound within tissues, within cells, within organelles, and extracellular eDNA both freely suspended and particle bound) introduces another level of complexity. While the dominant size class of eDNA bearing particles for some macro-organisms is known (Turner et al. 2014), it remains to be seen whether this profile is

consistent among organisms, across habitats, and under varying environmental conditions. Differential decay and production rates may be directly related not only to the driving mechanisms of production (e.g., sloughing rates or hydraulic shear stress) and decay (e.g., rates of enzymatic hydrolysis or ultraviolet damage), but also to the state of the eDNA(e.g., bound within cells, attached to particles, or incorporated in biofilms).

Quantification biases can also arise from inhibition of recovery and amplification, from variability of detection limits across organisms and marker sets, and from contamination during collection, extraction, amplification, and handling of samples. Rapid advances in technique such as separation of high copy and low copy materials (Champlot et al. 2010) and development of other best practices are imperative to overcoming these biases (Goldberg et al. 2015).

Advances in molecular technology will also provide higher resolution measurement of target eDNA concentrations. For example, technologies such as rapid whole genome sequencing can increase efficiency in two ways. First, full genome and/or mitogenome sequencing from vouchered specimens will allow for better location of diagnostic base pair sequence differences between target and non-target organisms, effectively enabling better marker design. Second, whole metagenome sequencing from environmental samples may be able to detect and quantify target organism DNA directly through matching of operational taxonomic units (e.g., Evans et al. 2015). Also, amplification techniques such as digital droplet PCR (Hindson et al. 2013, Doi et al. 2015), which separates each well on a PCR plate into 10,000 or more droplets – each with a single PCR reaction, may provide lower detection limits. By running massively parallel single reactions in droplets, target eDNA can be detected at lower copy numbers and with higher precision than what is possible with conventional qPCR methods. Also, due to the properties of the Poisson distribution of detect/non-detect results, standard curves from amplicons or cloned plasmids are not necessary. Development of assays with multiplex reactions having internal positive controls in each well (e.g., Turner et al. 2015) will also provide a higher level of confidence in

non-detect results, because it can be determined whether non-detections are attributable to inhibition of amplification

Now that best practices for working with eDNA are being established, more emphasis can be devoted to the questions that eDNA analyses could investigate. Future studies should focus on basic questions such as where is the target eDNA in the environment? What state is it in? What proportion is bound, unbound, attached, free floating, etc.? Where and how does it travel? Are there distinct patterns across habitats, environmental compartments, seasons, or organisms? How do production and loss mechanisms compare across those same distinctions? What are the drivers of net eDNA concentrations in the environment, and are the inflection points (i.e., when production outpaces loss or vice versa) predictable? As part of the development of standardized techniques, some basic applied questions will also be vital to establishment of effective, standardized monitoring programs. For example, given the patchy distribution of target material, is it better to take more small volume samples or fewer large volume samples? Are there particular places within a monitoring network or habitat that might be more important to sample than others (e.g., riffles downstream from pools where fish spawn, sediment in pools, surface films concentrated in flow vertices, etc.)? What are the practical limits of detection probability in large waterbodies versus small waterbodies or for large biomass populations versus small biomass populations?

To address these types of questions, new approaches should examine multiple environmental compartments, such as water columns, surficial sediments, attached biofilms, surface microlayers, and transparent exopolymer particles. Transport and fate among these compartments are likely quite different, but some overall trends may exist. For example, certain habitats may be dominated by settling of large particles from the water column to sediments, while others may be dominated by shearing and mixing of target material in the water column or formation of floating slicks of material. Additional time trials should help discern the relative contributions of settling and resuspension over time. Phenology of organisms likely also contributes to variable eDNA concentrations, with potential for seasonal increases

in eDNA production rates associated with spawning (e.g., direct release of gametes, sloughing due to physiochemical changes, sloughing due to territorial aggression). Changes in degradation rates associated with seasonal patterns of ultraviolet light intensity or temperature mediated differences in enzyme activity are likely also important. As suggested by results from this study, a seasonal index period may be more informative than year round monitoring due to relative differences in production and decay rates. Index periods are common in other types of biological assessments to help maximize signal to noise ratios and maintain comparability between samples (Cuffney et al. 1993, Barbour et al. 1999).

Predictive modeling of eDNA concentrations is another logical step in the development of eDNA as a monitoring tool. Large portions of the variability in eDNA concentrations observed in this study were attributable to temperature and biomass effects. However, consistent prediction likely requires more direct measurement of the mechanisms driving decay - hydrolysis (e.g., enzyme activity or concentration) and ultraviolet light. For prediction in real world systems, spatial modeling will also be important. The conceptual framework of Leff et al. (1992) describing downstream transport of cellular and genetic material suggests that particle transport models may also be useful tools for determining pathways and hotspots of target eDNA in complex lotic systems. For example, particle transport models may provide a better understanding of hydraulic transport of eDNA in aquatic systems, which may help identify locations where target eDNA might concentrate due to flow fields, hydraulic residence times, or other hydraulic or spatial phenomena. Recent research has also shown that enzymatic hydrolysis may have fractal kinetics both within cells (Vasilescu et al. 2013) and in marine sediments (Arndt et al. 2013), suggesting that environments with variable diffusion rates and complex matrices may nonetheless produce predictable decay patterns consistent with power law dynamics. Preliminary evidence from eDNA degradation in sediments in this paper supports the hypothesis that such power relationships may also exist for fish eDNA in aquatic sediments. Further modeling in this regard may provide further insight into degradation dynamics of macro-organismal eDNA in the environment. Long-term

monitoring of target eDNA concentrations in multiple environmental compartments could also provide sufficient datasets to develop and calibrate predictive models.

By increasing prediction capability of the location, movement, and concentration of eDNA, researchers will be able to implement eDNA as a quantitative tool for environmental monitoring with applications from site occupancy and historical range determination, to population monitoring and seasonal observation. With sufficient precision and accuracy, eDNA based quantification may also provide threshold indicators for management actions, such as removal of invasive species or protection, restocking, or relocation of endangered species. Despite the technical obstacles to quantification of macro-organisms using eDNA, rapid advances in both techniques and technology suggest environmental DNA will be an important tool for environmental monitoring in the future.

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APPENDIX A -- Fish environmental DNA is more concentrated in aquatic sediments than surface water

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Title: Fish environmental DNA is more concentrated in aquatic sediments than surface water.

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Conceived and designed the experiment: CRT RCE KLU. Developed field methods: RCE CRT.

Developed lab methods: CRT KLU. Performed the experiment: CRT RCE KLU. Analyzed the data:

CRT. Contributed reagents/materials: CRT RCE. Wrote the paper: CRT RCE KLU.

Highlights:

- Fish eDNA was 8 to 1800 times more concentrated in sediment than water.
- Fish eDNA remained detectable after more than 3 months in sediment but not water.
- Aqueous and sedimentary concentrations of fish eDNA were positively correlated.
- Sedimentary eDNA may allow retrospective genetic monitoring of fish.
- Avoid sedimentary eDNA when seeking inferences about very recent site occupancy.

Abstract: Genetic identification of aqueous environmental DNA (eDNA) provides site occupancy inferences for rare aquatic macrofauna that are often easier to obtain than direct observations of organisms. This relative ease makes eDNA sampling a valuable tool for conservation biology. Research on the origin, state, transport, and fate of eDNA shed by aquatic macrofauna is needed to describe the spatiotemporal context for eDNA-based occupancy inferences and to guide eDNA sampling design. We tested the hypothesis that eDNA is more concentrated in surficial sediments than in surface water by measuring the concentration of aqueous and sedimentary eDNA from an invasive fish, bigheaded Asian carp (*Hypophthalmichthys* spp.), in experimental ponds and natural rivers. We modified a simple, low-cost DNA extraction method to yield inhibitor-free eDNA from both sediment and water samples. Carp eDNA was 8 to 1800 times more concentrated per gram of sediment than per milliliter of water and was detected in sediments up to 132 days after carp removal - five times longer than any previous reports of macrobial eDNA persistence in water. These results may be explained by particle settling and/or retarded degradation of sediment-adsorbed DNA molecules. Compared to aqueous eDNA, sedimentary eDNA could provide a more abundant and longer-lasting source of genetic material for inferring current-or-past site occupancy by aquatic macrofauna, particularly benthic species. However, resuspension and transport of sedimentary eDNA could complicate the spatiotemporal inferences from surface water sampling, which is currently the predominant eDNA-based approach. We discuss these implications in the context of conservation-oriented monitoring in aquatic ecosystems.

Keywords: environmental DNA; water; sediment; fishes; rare species; genetic monitoring

Abbreviations:

CTAB - cetyl trimethyl ammonium bromide

eDNA - environmental DNA

IPC - internal positive control

KUFS - University of Kansas Field Station

NCBI - National Center for Biotechnology Information

PCR - polymerase chain reaction

qPCR - quantitative real-time PCR

1. Introduction

Conservation and management of biodiversity relies on effectively monitoring rare or patchily distributed species across large areas. However, directly observing and identifying such species is often difficult and expensive (Bogich et al., 2008). Less direct methods such as camera traps, acoustic surveys, and noninvasive genetic sampling can be easier, cheaper, and less harmful (Beja-Pereira et al., 2009; Jewell, 2013; Stanley and Royle, 2005). For rare aquatic macrofauna, aqueous environmental DNA (eDNA) sampling is a recent extension of noninvasive genetics where a sample of bulk environmental material (i.e., water or suspended solids) is assayed for the presence of species-identifying DNA fragments without isolating target organisms or their parts from the sample (Ficetola et al., 2008). This method provides inferences about occupancy (Dejean et al., 2012) and abundance (Pilliod et al., 2013; Takahara et al., 2012; Thomsen et al., 2012b) that are simple and inexpensive to obtain, once robust sample collection and assay protocols are established (Hayes et al., 2005; Wood et al., 2013). Importantly, development of robust sample collection and assay protocols is difficult, expensive, and time-consuming, making eDNA methods less valuable for abundant organisms that are easily observed and identified by direct methods.

Determining how well eDNA can serve as a proxy for directly observing organisms is an area of active research that will influence how eDNA methods should be applied to biological conservation (Foote et al., 2012; Lodge et al., 2012; Pilliod et al., 2013, 2014). Guidance is available from other fields that make inferences about organisms based solely on indirect genetic evidence from environmental samples. These

include microbiology (Liang and Keeley, 2013), forensics (van Oorschot et al., 2010), paleogenetics (Knapp et al., 2012), fecal pollution tracking (Caldwell et al., 2011), and agricultural transgene monitoring (Nielsen et al., 2007). Across these fields, three major features differentiate detection of eDNA from detection of organisms: **contamination**, **time**, and **space**. First, contaminating DNA molecules from the target organism(s) can enter eDNA samples at any point in the sampling process, from preparation of supplies to genetic assay (Kowalchuk et al., 2007). High-concentration DNA such as polymerase chain reaction (PCR) products and fresh tissue produce the greatest risk of contamination that cannot be objectively distinguished from real eDNA detection (Champlot et al., 2010). Second, eDNA can persist for days to thousands of years, depending on starting concentration and degradation conditions (Levy-Booth et al., 2007). Third, organisms can move long distances from where they shed eDNA and physical forces can move eDNA far from its organismal source (Douville et al., 2007). These features of eDNA detection create uncertainty whose characterization and appropriate use requires better understanding of eDNA in four domains: **origin**, **state**, **transport**, and **fate**. The **origin** of eDNA describes its physiological sources, commonly hypothesized to be feces, urine, gametes, skin, and decomposition (Caldwell et al., 2011). The **state** of eDNA describes its mutable physical forms, such as particle-bound or freely dissolved DNA molecules (Turner et al., 2014). The **transport** of eDNA describes its movement after leaving the source organism, including settling and downstream flow in water (Deiner and Altermatt, 2014). The **fate** of eDNA describes its transformation from intact genomic DNA within living cells into extracellular DNA fragments too small for identification (Barnes et al., 2014). Discoveries in these four domains are beginning to establish the spatial and temporal context for eDNA-based inferences and guide eDNA sampling design. eDNA surveys can effectively inform conservation efforts only when this uncertainty information is used.

In the present study we aimed to gain new understanding about the **transport** of eDNA shed by fish, specifically its relative concentration in two alternative locations: surface water and surficial sediments.

Comparing eDNA concentration in water and sediment is valuable because aquatic particles readily move between them (Leff et al., 1992) and because transport and degradation of aquatic particles can differ substantially between them (Pietramellara et al., 2009). Furthermore, surface water sampling is the primary sampling strategy for eDNA surveys of aquatic macrofauna and the spatiotemporal context of its inferences could be linked to sedimentary eDNA by settling and resuspension dynamics. Depending on eDNA concentration and persistence, sediment samples might be more useful than water samples for monitoring rare macrofauna, particularly benthic species. Previous work on the **origin** and **state** of macrofaunal eDNA led us to hypothesize that fish eDNA concentration is higher in sediment than water. Feces are a major source of aqueous macrofaunal eDNA, because they are regularly expelled in large quantities and can contain high concentrations of DNA (Caldwell et al., 2011; Corse et al., 2010). Most animal feces rapidly sink (Robison and Bailey, 1981; Saba and Steinberg, 2012; Wotton and Malmqvist, 2001) and many eDNA-bearing particles of different origins likely sink as well. For example, Turner et al. (2014) recently demonstrated that most of the aqueous eDNA-bearing particles for Common Carp (*Cyprinus carpio*) are too large to stay suspended indefinitely (>1 μm; Maggi, 2013). Settling should lead to higher concentrations of fish eDNA in sediment than water.

Previous work on the **transport** and **fate** of microbial eDNA also informed our hypothesis. eDNA concepts and terminology originated in microbiology (Ogram et al. 1987) yet an important distinction must be made between microbial and macrobial eDNA. Microbial eDNA includes both intraorganismal and extraorganismal eDNA from microorganisms such as bacteria. Water, sediment, and virtually any environmental material contains abundant living microbes with active, replicating DNA (intraorganismal eDNA), along with some extracellular DNA from dead microbes (extraorganismal eDNA) (Corinaldesi et al., 2005). In contrast, macrobial eDNA is primarily from dead or dying matter (extraorganismal eDNA), especially for large animals (Andersen et al., 2012). Thus microbial extracellular eDNA is more comparable to macrobial eDNA but still not completely analogous given animal multicellularity and the

primary use of mitochondrial DNA (mtDNA) in macrobial eDNA studies. Macrobial eDNA that is free of cellular and mitochondrial membranes is most analogous to microbial extracellular eDNA, and the term extramembranous comprises both. Recent studies have shown that microbial extracellular DNA is found in higher concentrations in sediment than the overlying water column (Corinaldesi et al., 2005) and that microbial DNA from the water column can progressively accumulate in sediments (Corinaldesi et al., 2011). These findings suggest that settling and/or preservation of extramembranous DNA could cause fish eDNA to be more concentrated in aquatic sediment than in water.

To test our hypothesis, we measured the concentration of eDNA from an invasive fish in surface water and surficial sediment from experimental ponds and natural rivers. We adapted a simple, low-cost DNA extraction method to produce sedimentary and aqueous eDNA that was free of polymerase chain reaction (PCR) inhibition. Comparison of the sedimentary and aqueous reservoirs of fish eDNA provides a more comprehensive understanding of the processes that affect observed eDNA concentrations, potentially providing further insight to inferences made when using eDNA as an indirect detection method. To our knowledge, this comparison represents the first evaluation of sediments as a source material for eDNA-based monitoring of aquatic macrofauna.

2. Material and methods

2.1 Target species

One of the first and the largest conservation programs with eDNA-based monitoring as a central instrument is focused on bigheaded Asian carp (*Hypophthalmichthys* spp., hereafter bigheaded carp) (USACE, 2013; USACE et al., 2013; Jerde et al., 2013; USFWS, 2013). Bigheaded carp were imported to North America as two separate species, Bighead Carp (*Hypophthalmichthys nobilis*) and Silver Carp

(*Hypophthalmichthys molitrix*). However, since establishing in the Mississippi River basin, *Hypophthalmichthys* hybridization is widespread, including fertile post-F1 hybrids and F1 hybrid frequency estimates as high as 73% for the silver carp morphotype (Lamer et al., 2010; Stuck, 2012). This hybrid swarm may be developing into a new species complex (Lamer et al., 2010) as the genus expands its range northward (Kolar et al., 2007; USGS, 2013). These large planktivorous fish threaten native fisheries due to their dietary overlap with native filter feeders (Sampson et al., 2008) and their tendency to reach high abundance and biomass in their invaded range (Chapman and Hoff, 2011). These characteristics have implicated bigheaded carp in the decline of at least two commercially important fish species in the Mississippi basin, Gizzard Shad (*Dorosoma cepedianum*) and Bigmouth Buffalo (*Ictiobus cyprinellus*) (Irons et al., 2007). Recent analyses predict that small introductions of bigheaded carp could become established (Cuddington et al., 2013) and cause significant ecological and economic harm in many coastal embayments, wetlands, and tributaries of the Laurentian Great Lakes (Cooke and Hill, 2010; Cudmore et al., 2012).

2.2 Pilot sampling

The primary sampling design described in this study was informed by pilot sampling conducted earlier. Here we describe the details of pilot sampling that differed from the primary sampling design. Pilot sampling used four ponds located at the United States Geological Survey (USGS) Columbia Environmental Research Center (CERC) in Columbia, Missouri, USA (LatDD/LonDD: 38.911980, -92.276825). The earthen ponds measured 37 m by 21 m with a maximum depth of 1 m. Each pond contained multiple bigheaded carp until July 2011 when they were emptied of fish, drained of water, and renovated with earthmoving equipment. Renovation included scraping and removing soft surface sediments from the clay substratum and lining the banks with gravel. At the time of sampling on October 21, 2011 three ponds had been partially filled with well water to approximately 0.3 m for the first time

since renovation and one pond was still empty. We collected and filtered five 2 L water samples from the three partially filled ponds following the protocol of Jerde et al. (2013). We collected five sediment samples from all four ponds by hand using sterile 50 mL tubes. Sampling containers filled with sterile water were included as collection negative controls for water and sediment. Aqueous eDNA was extracted following Jerde et al. (2013) and sedimentary eDNA was extracted from 5 g of each sediment sample using PowerMax Soil DNA Isolation Kits (MO BIO Laboratories, Carlsbad, California, USA). pGEM-3Z plasmid (Promega, Madison, Wisconsin, USA) was added to the first extraction solution at 0.02 ng μL^{-1} as internal positive control (IPC) DNA for PCR inhibition testing (Coyne et al. 2005). We tested all eDNA extracts for PCR inhibition using a pGEM-specific IPC assay (Coyne et al. 2005) with pGEM amplification providing qualitative evidence for a lack of inhibition. Inhibited extracts were diluted until pGEM amplified. All other details, including qPCR assay for bigheaded carp eDNA, were as described below.

2.3 Sampling sites

We sampled both experimental ponds and natural rivers containing bigheaded carp. The experimental ponds were located at the University of Kansas Field Station (KUFS) in Lawrence, Kansas, USA (LatDD/LonDD: 39.047452,-95.191526). Ten earthen ponds had been stocked with at least one bigheaded carp on May 30, 2012, and one additional pond (Pond 311) contained no fish and served as a negative control site. Bigheaded carp had never been present at KUFS prior to May 30, 2012 and quantitative real-time PCR (qPCR) testing of the well water and sediment from each pond prior to stocking detected no bigheaded carp eDNA (data not shown). The ten ponds with bigheaded carp also contained at least one Bluegill Sunfish (*Lepomis macrochirus*), Redear Sunfish (*Lepomis microlophus*), White Crappie (*Pomoxis annularis*), Common Carp (*Cyprinus carpio*), and Grass Carp (*Ctenopharyngodon idella*). The number of bigheaded carp stocked ranged from zero to 46 per pond and the number of total fish ranged

from zero to 57 per pond. The density of bigheaded carp stocked per pond ranged from zero to 0.016 g L^{-1} and the density of total fish ranged from zero to 0.02 g L^{-1}. Between June 4 and June 5, 2012 the only bigheaded carp stocked in KUFS pond 321 died and was removed within 24 hours of death. The ponds measured 21 m^2 with a maximum depth of 3 m and were filled to approximately 450 m^3 with KUFS well water. After filling there was no water flow through the ponds. Water and sediment samples were collected on October 8-15, 2012. The experimental pond study at KUFS was conducted in accordance with a protocol for field research on live vertebrates (protocol number 211-01) approved by the University of Kansas Institutional Animal Care and Use Committee.

The natural sites were located on the Wabash River in West Lafayette, Indiana, USA, the Kansas River near Desoto, Kansas, USA, and the Wakarusa River below Clinton Reservoir near Lawrence, Kansas, USA. Bigheaded carp have been captured at each of these sites (USGS, 2013).

The Wabash River is a large, unchannelized tributary of the Ohio River with a 1924-2012 mean annual flow between 46 and 352 $m^3 s^{-1}$. The Wabash River stretches 810 km and drains approximately 103,500 km^2 of Ohio, Indiana, and Illinois. Our Wabash River sampling site (LatDD/LonDD: 40.430281,-86.897993) was a borrow pit connected to the main river channel and located approximately 600 m upstream of the nearest United States Geological Survey (USGS) gaging station (USGS 03335500), which reported a mean daily flow of 78 $m^3 s^{-1}$ on the day of sampling (November 13, 2013).

The Kansas River is a large, sand-bottom prairie river characterized by a relatively wide, shallow channel (100 to 500 m wide, 0.5 to 3 m deep) and flood control levees on both banks with a 1918-2013 mean annual flow between 38 and 866 $m^3 s^{-1}$. The Kansas River stretches 283 km and drains approximately

155,000 km^2 of Kansas, Colorado, and Nebraska. Our Kansas River sampling site (LatDD/LonDD: 38.984901, -94.97385) was approximately 800 m upstream of the nearest USGS gaging station (USGS 06892350), which reported a mean daily flow of 27 $m^3 s^{-1}$ on the day of sampling (November 26, 2013).

The Wakarusa River is a relatively narrow, shallow (15 to 20 m wide, 0.5 to 2 m deep) tributary of the Kansas River that spans 130 km and drains approximately 1100 km^2 of eastern Kansas. It has a 1930-2013 mean annual flow between 0.3 and 21 $m^3 s^{-1}$. The lower Wakarusa is constrained by incision and dominated by outflows from a large reservoir (Clinton Lake, 28 km^2) with peak daily flows ranging from 17 to 227 $m^3 s^{-1}$. Our Wakarusa River sampling site (LatDD/LonDD: 38.928506, -95.321393) was 800 m downstream from the reservoir outfall. The nearest USGS gaging station (USGS 06891500; approximately 8 km downstream of the sampling site) reported a mean daily flow of 0.15 $m^3 s^{-1}$ on the day of sampling (December 2, 2013).

2.4 Sample collection, preservation, and storage

Water samples were collected following the protocol described by Ficetola et al. (2008). We submerged a sterile 50 mL centrifuge tube slightly below the water surface, allowing it to fill with 15 mL of surface water (measured with the tube graduations), added 1.5 mL of 3M sodium acetate and 33.5 mL absolute ethanol, and then stored the tube on ice for 10 to 120 min until it could be frozen (-20ºC). At KUFS we collected three water samples from the shore at positions chosen by randomized selection from the entire shoreline divided into 20 sections. At the natural sites we collected three water samples from a boat along an approximate transect across the river. At each site we included a 'collection negative control' that consisted of a 50 mL tube containing 15 mL of tap water. This negative control tube was transported to the site alongside sample tubes and was treated as a sample from that point on. All samples were driven or shipped overnight on dry ice to the University of Notre Dame, and stored at -80ºC until eDNA extraction.

At every site, we collected all water samples prior to sediment sampling in order to avoid collecting eDNA in the water column that may have recently been resuspended from the sediment by our sediment collection. This precaution prevented any within-site pairing of water and sediment samples.

Our sediment collection method was modified from a method originally developed for collecting sediment diatoms (USEPA 2007). Sediment samples were collected using Wildco (Yulee, Florida, USA) hand corers and Wildco K-B corers with 5 cm internal diameter, 51 cm long stainless steel core tubes. We collected three samples along an approximate transect across the pond or river. At the Wakarusa River, outflow from the Clinton Reservoir dam had previously scoured away most of the unconsolidated sediment and we were able to collect only two sediment samples. For each sample we inserted a clear plastic liner tube into the corer and vertically dropped the corer from a small boat. The hand corer was fitted with additional weight to increase the sediment penetration depth. After gently pulling the corer to the surface, we took precautions to minimize disturbance of the sediment-water interface, including submerged plugging of the bottom end of the corer tube with a sediment core extruder, slowly extruding the core upward through the liner tube, and carefully pipetting the last few milliliters of water from atop the sediment core without extruding it past the top end of the liner tube (Glew et al., 2001). We collected 5 mL of wet surficial sediment from the top 2 cm of the sediment core using a 1 teaspoon measuring spoon and transferred this to a 50 mL centrifuge tube containing 10 mL of cetyl trimethyl ammonium bromide (CTAB) buffer (Coyne et al., 2006, 2001). The only exception to this protocol was at the Kansas River site, where sand-dominated sediments were not sufficiently cohesive to maintain core integrity. Thus Kansas River sediment samples were collected by hand in shallow water (~0.75 m) from the top 2 cm of the sediment surface with a sterile 50 mL centrifuge tube. The wet weight of each sample was measured by weighing the sample in its tube and subtracting off the previously measured weight of the tube and CTAB. Sediment samples from the Kansas and Wakarusa rivers were added to empty centrifuge tubes, stored on ice for 10 to 120 min, frozen (-20^{o}C), and shipped overnight on dry ice to the University

of Notre Dame where they were thawed, weighed, and preserved in CTAB immediately before eDNA extraction. All other samples were immediately preserved in CTAB, stored on ice for 10 to 120 min, weighed, frozen (-20ºC), driven on dry ice to the University of Notre Dame, and stored at -80ºC until eDNA extraction.

In between collection of each sediment sample, the corers, liner tubes, nosepieces, extruders, spoon, cables, and ropes were treated with a 4-step decontamination process. First, most visible sediments were rinsed and scrubbed into the pond or river water. Second, all equipment was submerged in solution of hot tap water, 10% bleach, and detergent, then scrubbed until all visible sediment traces were removed. Third, all equipment was submerged in solution of tap water and 10% bleach for 10 minutes. Fourth, all equipment was rinsed in tap water. In between sites we decontaminated field equipment (boat, waders, boots, etc.) by scrubbing away all visible sediment traces and spraying exposed surfaces with a solution of tap water and 10% bleach. We collected two negative control samples at each site to test for contamination during sampling. The 'corer negative control' consisted of 5 mL of water collected below an internal/external rinsing of a decontaminated and reassembled corer (corer, liner tube, nosepiece) and added to 10 mL of CTAB in a 50 mL centrifuge tube. The 'collection negative control' consisted of swirling the decontaminated measuring spoon in 10 mL of CTAB in a 50 mL centrifuge tube. Upon creation in the field these negative controls were treated as samples from that point on.

2.5 eDNA extraction and purification

DNA extraction was performed in a strictly pre-PCR laboratory separate from our post-PCR laboratory. During extraction we added an 'extraction negative control' to every batch of samples. This consisted of an empty 50 mL centrifuge tube containing 5 or 15 mL of autoclaved reverse osmosis water, which was

subsequently treated as a sample. Including separate extraction and collection negative controls allowed us to distinguish between collection-derived and extraction-derived contamination, if any were detected.

Dissolved and suspended particulate matter was precipitated and pelleted from the water samples by 35 min centrifugation at 6ºC and 3220g. eDNA was extracted from the pellet using a CTAB protocol (Coyne et al., 2005, 2006, 2001), and the final aqueous eDNA pellet was re-suspended in 100 µL of 1X TE Low EDTA buffer (USB Corporation, Cleveland, Ohio, USA) and stored at 4ºC until qPCR assay (Jerome et al., 2002). For sediment samples, we modified the CTAB extraction protocol of Coyne et al. (2005, 2006, 2001). A step-by-step description of our protocols is provided in Supplementary Appendix A1. The final sedimentary eDNA pellet was re-suspended in 1 mL of 1X TE Low EDTA buffer. At this stage we measured PCR inhibition on a subset of 27 sediment samples using the internal positive control assay described below. Results of this testing identified PCR inhibition in some samples (see Results) thus 200 µL of eDNA extract from all 27 samples was further purified using a OneStep Inhibitor Removal Kit (Zymo Research, Irvine, California, USA), according to the manufacturer's instructions. Testing of these purified extracts showed no evidence of PCR inhibition (see Results) thus all remaining sediment samples were extracted, purified, and tested for inhibition accordingly.

2.6 eDNA quantification

We measured bigheaded carp eDNA concentration in each eDNA extract using the Minor Groove-Binding (MGB) hydrolysis probe qPCR assay described in Turner et al. (***in review***), which has a 95% limit of detection of 30 copies reaction^{-1}. This assay targets a 100 base pair (bp) section of the mitochondrial control region (D-loop) of bigheaded carp and uses a FAM-labeled hydrolysis probe. We performed all reactions on a Mastercycler ep realplex2 S thermocycler (Eppendorf, Hauppauge, New York, USA) with the following reaction conditions: 50ºC for 2 min, 95ºC for 10 min, and 55 2-step cycles

of 95ºC for 15 sec and 60ºC for 1 min. Fluorescence data collection using the FAM filter (520 nm) occurred during the 60ºC step. We performed 20 µL sextuplicate reactions using 10 µL of TaqMan Environmental Master Mix 2.0 (Life Technologies, Carlsbad, California, USA), final primer concentrations of 300 nM each, a final probe concentration of 200 nM, and 4 µL of eDNA extract. To minimize variation between qPCR replicates (technical replicates) caused by imperfect pipetting of small eDNA extract volumes (Ellison et al., 2006), we combined eDNA extract and master mix (all other reagents) for six reactions into one tube then dispensed to six plate wells using an electronic repeating pipette (Xplorer 5-100 µL, Eppendorf). All liquid handling for qPCR used low bind tubes and low bind aerosol barrier pipette tips (Ellison et al., 2006) and each qPCR plate included two qPCR negative control reactions (no template controls - NTCs). qPCR setup was performed inside of an AirClean 600 dead air box with ultraviolet (UV) light (AirClean Systems, Creedmoor, North Carolina, USA) that was decontaminated with 15 minutes of UV irradiation after every use. Sealed qPCR plates were carried from the pre-PCR laboratory to the post-PCR laboratory for thermocycling.

We used a copy number standard curve made of complete D-loop (1022 bp) (Liu and Chen, 2003) PCR amplicon from tissue-derived Silver Carp DNA that was quantified using a Qubit fluorometer and the Qubit dsDNA High Sensitivity kit (Life Technologies). Standard curve DNA weight was converted to DNA copies using the median double-stranded molecular weight of the 95% consensus 1022 bp amplicon sequence from all Silver Carp mitogenomes on GenBank (635 518 g $mole^{-1}$) as calculated by OligoCalc version 3.26 (Kibbe, 2007). Each qPCR plate included a five point standard curve from $3*10^4$ copies $reaction^{-1}$ down to 3 copies $reaction^{-1}$. The fluorescence threshold for each plate and the fluorescence baseline for each reaction were determined using default settings of the Eppendorf realplex software version 2.2 (Noiseband and Automatic Baseline, respectively). Every amplification profile was visually examined to confirm exponential amplification. To provide additional verification of qPCR assay specificity, beyond the in silico (NCBI GenBank), in vitro (tissue-derived DNA from non-target species),

and in situ (eDNA from sites with target and non-target species) testing described in Turner et al. (***in review***), we purified (ExoSAP-IT, USB Corporation) and Sanger sequenced (ABI 3730xl, Applied Biosystems) qPCR product from at least one water sample and one sediment sample for every site.

We tested every eDNA extract for PCR inhibition using an internal positive control (Universal Exogenous qPCR Positive Control for TaqMan Assays, Yakima Yellow-BHQ-1 Probe Kit, Eurogentec, San Diego, California, USA). This internal positive control (IPC) assay was used in duplex with the bigheaded carp assay by including 2 µL of IPC mix and 0.4 µL of IPC DNA in the 20 µL reactions. Prior to its application in this study we conducted tests confirming the absence of cross-reactivity between IPC and bigheaded carp assays and the stability of Cq values when the two assays were run separately or in duplex. qPCR amplification of the IPC DNA was measured with fluorescence data collection using the VIC filter (550 nm) during the 1 min. 60ºC thermocycling step. For each qPCR plate we used the average IPC quantification cycle (Cq) from the reference reactions (standard curve and NTCs) as the expected IPC Cq in order to calculate an IPC ΔCq value (IPC ΔCq = expected IPC Cq - observed IPC Cq) for every eDNA reaction. Following the protocol of Hartman et al. (2005), we used an IPC ΔCq value of three cycles as the threshold defining PCR inhibition. This threshold was supported by our observation that the maximum range of IPC Cq values in the reference reactions on any qPCR plate was 2.8 cycles.

2.7 Data analysis

Following the recommendation of Ellison et al. (2006) for qPCR with low level DNA, we calculated concentrations for each reaction, assigning zero concentration to non-detect reactions and averaging concentration across the six technical replicates for each eDNA extract. In three reactions the measured reaction copy number was slightly below one, so we rounded all reaction copy numbers up to the next largest integer. Final aqueous eDNA concentrations were expressed in copies mL^{-1}, and final sedimentary

eDNA concentrations were expressed in copies g^{-1}. To test for a significant difference in concentration between sedimentary and aqueous eDNA, we used the Wilcoxon signed-rank test because the data exhibited non-normal error distribution. To test for a significant relationship between sedimentary and aqueous eDNA concentrations we used a generalized linear model (GLM). Because the data were positive-only with positively-skewed errors we used the Gamma distribution and log link function (Crawley, 2005; Zuur et al., 2010). To compare detection probability (i.e., diagnostic sensitivity) between sedimentary and aqueous eDNA, we calculated the proportion of true positive samples and the associated 95% confidence interval for a binomial probability using the Wilson score method (Newcombe, 1998). All statistical analyses used an alpha level of 0.05 and were performed in R version 3.0.1 (R Core Team, 2014).

3. Results

Aqueous eDNA from pilot sampling showed no evidence of PCR inhibition but sedimentary eDNA extracts required 10-fold dilution before the pGEM-IPC amplified. For primary sampling all aqueous eDNA samples produced an IPC ΔCq value <3 cycles whereas 13 of the initial 27 sedimentary eDNA extracts produced an IPC ΔCq value ≥3 cycles (range: 3.2 to 16.5). Purification of sedimentary eDNA extracts using the OneStep Inhibitor Removal Kit reduced all IPC ΔCq values to <3 cycles (range: -1.3 to 1.4), demonstrating that our modified versions of the Coyne et al. (2005, 2006, 2001) protocol for eDNA extraction effectively removed PCR inhibitors from both sediment and water (Burnet et al., 2012; Hartman et al., 2005). Our experimental, field, and laboratory controls showed no contamination. No bigheaded carp eDNA was detected in sediment or water from the KUFS negative control pond (Pond 311; no fish present), indicating that pond maintenance and sampling protocols successfully prevented cross-contamination among experimental ponds. No bigheaded carp eDNA was detected in corer negative controls or collection negative controls, demonstrating the effectiveness of our 4-step decontamination

protocol for sediment core sampling. All extraction controls and NTCs also showed no detection of bigheaded carp eDNA, indicating that our contamination precautions were sufficient at every step, from sample collection to qPCR assay. The range of qPCR efficiency across the entire study, calculated from the slope of standard curves, was 96% to 104% and the range of standard curve R^2 values was 0.963 to 1.00. All Sanger sequenced qPCR products matched the target amplicon from bigheaded carp.

Pilot sampling of four renovated CERC ponds with no bigheaded carp presence in the preceding three months yielded no detection of aqueous eDNA but did detect sedimentary eDNA in two ponds - one containing water and one that was empty. These results motivated the primary sampling design.

At every primary sampling site, bigheaded carp eDNA was more concentrated per g of sediment than per mL of water (8 to 1846 fold; Table 1) and overall sedimentary eDNA concentration was significantly higher than aqueous eDNA concentration (P=0.0002). By using the conventional units of weight for sediment and volume for water these concentration comparisons implicitly assume the equivalence of 1 mL and 1 g of water. All sediment samples were collected with a 5 mL scoop so sedimentary eDNA concentrations could also be expressed per mL of sediment. However, sediment sample weight ranged from 5.5 to 10.9 g so the use of volume would only produce higher sedimentary eDNA concentrations, leaving the overall results unchanged.

Sedimentary and aqueous eDNA concentration were positively correlated when analyzed across all primary sampling sites (P=0.001; Figure 1) and within the KUFS ponds (P=0.002; Figure 1). A correlation test was not performed on the natural sites alone because only three were sampled.Aqueous eDNA sampling failed to detect the presence of the bigheaded carp in one experimental pond, whereas

sedimentary eDNA sampling never failed to detect site presence (Table 1). Average detection probability (i.e., diagnostic sensitivity) across all primary sampling sites was 89% for sedimentary eDNA and 72% for aqueous eDNA, considering bigheaded carp present at the natural sites. This difference was not statistically significant based on overlapping 95% confidence intervals. Bigheaded carp eDNA was detected in sediment from one experimental pond 132 days after the single bigheaded carp was removed, whereas no target eDNA was detected in the water from that pond (KUFS pond 321; Table 1).

4. Discussion

As we hypothesized, concentrations of bigheaded carp eDNA were consistently higher in sediment than water. These results concur with reported differences between sedimentary and aqueous concentration of total extracellular DNA in microbiology studies (Corinaldesi et al., 2005; Dell'Anno and Corinaldesi, 2004; Pietramellara et al., 2009). Also, a recent comparison between sedimentary and aqueous eDNA of *Cyprinid herpesvirus 3*, a DNA virus that infects Common Carp, found 46-1238 times higher concentration in sediment than in the water column (Honjo et al., 2012). Thus, aquatic sediments appear to accumulate fish eDNA, viral eDNA shed by fish, and extracellular microbial eDNA. Although we demonstrated that net accumulation (i.e., deposition minus degradation and transport) of bigheaded carp eDNA is higher in sediment than water, the respective roles of degradation and transport remain to be determined. Few paired measurements of DNA degradation rate in water and sediment exist (Pietramellara et al., 2009), and evidence exists for both faster and slower DNA degradation rates in sediment compared to water (Corinaldesi et al., 2011; Dell'Anno and Corinaldesi, 2004; England et al., 2005, 2004). The focus of previous studies on extramembranous DNA (i.e., DNA molecules not protected by cellular, organellar, or viral membranes) limits comparison with naturally occurring fish eDNA, which could exist in multiple states along a continuum from whole living organisms (e.g., larvae) down to 'free' extramembranous DNA molecules not bound to other particles. We suspect that settling of fish eDNA-

bearing particles is the dominant process explaining the large accumulation of sedimentary eDNA we observed, but more research is needed to tease apart settling from degradation rate.

The other main result, that sedimentary eDNA lasted longer than aqueous eDNA, is linked to concentration differences because eDNA degradation generally follows an exponential decay pattern where higher starting concentration creates longer persistence (Barnes et al., 2014; Thomsen et al. 2012a). In pilot sampling we detected no bigheaded carp eDNA in water from three CERC ponds that were fishless for three months. Water from KUFS pond 321 also produced no detection 132 days after removal of its bigheaded carp. However, bigheaded carp eDNA was detected in sediment from ponds without bighead carp: KUFS pond 321, one of the three watered CERC ponds, and one CERC pond that was dry. These repeated observations of fish eDNA lasting 90+ days in sediment but not water are consistent with our finding that eDNA concentration was always higher in sediment than water. By comparison, previous studies of aqueous macrobial eDNA found a maximum persistence time of 25 days (Barnes et al., 2014; Dejean et al., 2011; Goldberg et al., 2013; Pilliod et al., 2014; Thomsen et al., 2012a, 2012b). The persistence we observed is not unusual in the context of literature on the fate of extraorganismal eDNA in soils and sediments (Pietramellara et al., 2009). For example, unfrozen lake sediment cores yielded fish eDNA from 3600 yr before present (BP; Matisoo-Smith et al., 2008) and mammal eDNA from 4800 yr BP (Giguet-Covex et al., 2014). Given the DNA-preserving properties of aquatic sediments we suspect the temporal window for surficial sedimentary eDNA extends much further than 132 days, which was the longest our study could observe. Sediments reduce biologically driven DNA degradation by adsorbing both DNases and DNA molecules (Levy-Booth et al., 2007; Pietramellara et al., 2009). Chemically driven DNA degradation (e.g., depurination) also appears to be reduced in aquatic sediments compared to terrestrial environments (Corinaldesi et al., 2008). Experiments measuring the degradation rate of aquatic macrofaunal eDNA in surficial sediments are needed to estimate the temporal window of eDNA persistence for diverse species and environments.

The concentration and persistence results collectively suggest a strategy for how aqueous and sedimentary eDNA from aquatic macrofauna should be used for biological conservation. First, sediment sampling may provide higher detection probability if water sampling - which is far easier to conduct - proves inadequate. A recent eDNA survey for invasive crayfish sampled mixtures of resuspended sediment and water, suggesting that more convenient surface water sampling failed for this benthic species (Tréguier et al., 2014). Interestingly, in our data the relative standard error (RSE=SE/mean) of within-site eDNA concentration estimates was high for both sediment and water in spite of the significantly higher average concentrations in sediment (Table 1). As discussed by Pilliod et al. (2013), RSE values over 20% indicate high spatial heterogeneity for both types of eDNA and recommend the use of larger amounts of water/sediment per sample and/or more than three samples per site. Taberlet et al. (2012) describe a spatially-integrated approach for sampling terrestrial soil eDNA that may be transferable to aquatic sediments. However, our persistence results present a major caution for sedimentary eDNA sampling in many conservation applications: surficial sediment provides detection of current-or-past occupancy (at least 132 days) whereas surface water provides detection of current-or-recent occupancy (up to 25 days). Conservation programs requiring data on recent occupancy should avoid sediments in favor of water sampling. Aqueous eDNA capture methods such as portable filtration (Goldberg et al., 2011; Pilliod et al., 2013) or continuous flow centrifugation (Zuckerman and Tzipori, 2006) can increase water sampling effort (i.e., water volume processed) relatively easily, which may mitigate the slightly lower detection probability we observed from water compared with sediment.

The high concentration and long persistence of fish eDNA in surficial sediments creates an opportunity for sediment resuspension to influence both the temporal and spatial scales of inference from aqueous eDNA (Bloesch, 1995; Douville et al., 2007; Graf and Rosenberg, 1997; Leff et al., 1992). We saw no

evidence of resuspension-derived aqueous eDNA in the three CERC ponds where it could have been identified after dewatering and refilling. However, since resuspension occurs when shear stress on the sediment bed exceeds a critical threshold value (Vanoni, 2006), studies monitoring for very recent occupancy should avoid sampling shallow lakes or ponds experiencing high bed shear stress conditions. For example, increased resuspension of sediment particles has been observed during or immediately after high wind events (Evans, 1994), in areas with significant wave action (Mian and Yanful, 2004), and in areas with substantial bed loads (the surficial sediments that are transported along the bed; Debnath et al., 2007). Similarly, results from a natural stream experiment with tracer bacteria seeded in sediments (Jamieson et al., 2005) suggest that water sampling in rivers during unusually high flow events would increase the chance of collecting old eDNA resuspended from sediment beds that are stable at lower flow. Human disturbance and transport of aquatic sediments should also be considered, including that caused inadvertently by scientists collecting eDNA samples. For example, cleaning mud from footwear between sites appears to be particularly important given high sedimentary eDNA concentration.

The low temporal resolution of sedimentary eDNA may be appropriate for conservation programs that can use information about current-or-past occupancy, such as retrospective genetic monitoring (Schwartz et al., 2007) of aquatic macrofauna for spatial distribution and historical range studies concerned with long-term site occupancy (Fernández et al., 2010; Provan et al., 2007; Wandeler et al., 2007). The abundance and persistence of sedimentary eDNA could benefit monitoring for species introductions or range shifts where the target species has no prior occupancy (Tréguier et al., 2014). Retrospective monitoring of macrofauna using sedimentary eDNA is well established for terrestrial sediments (Andersen et al., 2012; Haile et al., 2009), but analysis of aquatic sediments for macrobial eDNA has largely been limited to plants (Anderson-Carpenter et al., 2011; Boessenkool et al., 2013; but see Giguet-Covex et al., 2014; Matisoo-Smith et al., 2008; Naviaux et al., 2005). Our results suggest that fish eDNA in aquatic sediments may be a promising source of historical genetic material, although further research is

needed to evaluate the generality of our small study. Monitoring and managing biodiversity during the course of human population growth and ecosystem modification is a central goal of conservation biology, and aquatic animals are particularly vulnerable to extinction and difficult to monitor (Xenopoulos et al. 2005). The different temporal windows provided by sedimentary and aqueous DNA should facilitate, for example, determination of historical native range from sediment and seasonal occupancy from water, thereby enabling more effective conservation actions.

The positive correlation between sedimentary and aqueous eDNA likely represents a relationship with both downward and upward processes (Figure 2). Suspended particles (i.e., aqueous eDNA) may eventually sink downward and accumulate in the surficial sediment layer (i.e., sedimentary eDNA). Alternatively, large pieces of DNA-rich fish ejecta (e.g., feces; Caldwell et al., 2011; Corse et al., 2010) which have rapidly settled to the substratum (Robison and Bailey, 1981; Saba and Steinberg, 2012), may slowly disintegrate (Wotton and Malmqvist, 2001) and release smaller DNA-containing particles upward back into the water column through resuspension by turbulent flow and bioturbation (Bloesch, 1995; Graf and Rosenberg, 1997; Leff et al., 1992). These processes are common for many aquatic particles but have not yet been specifically studied with respect to eDNA. Factors such as water flow, substrate, wind, depth, stratification, and biota likely determine whether downward or upward processes dominate the link between aqueous and sedimentary eDNA. Better understanding of how these processes influence eDNA is needed if research continues to pursue aqueous eDNA concentration as an indicator of organism abundance or proximity (Pilliod et al., 2013; Takahara et al., 2012; Thomsen et al., 2012b).

Importantly, many of the sedimentary eDNA concentrations we measured would have been erroneous without use of an IPC assay sensitive to partial PCR inhibition. Even the use of commercial soil extraction and qPCR reagent kits specifically designed to mitigate inhibition did not guarantee success.

Measuring partial inhibition with IPC ΔCq led us to add the OneStep Inhibitor Removal Kit, which simply and affordably removed remaining inhibitors. Although the small-volume aqueous eDNA samples in this study showed no inhibition after CTAB extraction we have observed it for other samples, particularly from large volumes or water with high concentrations of algae or suspended sediment (CRT, unpublished data). In agreement with the MIQE guidelines (Bustin et al., 2009) and recent reviews (Hedman and Rådström, 2013) we recommend application of an IPC assay sensitive to partial PCR inhibition for all eDNA studies, especially when eDNA quantification is attempted.

In conclusion, we adapted a simple, low-cost extraction method to recover inhibitor-free eDNA from both sediment and water samples and showed that bigheaded carp eDNA is more concentrated in sediment. Sedimentary eDNA was a slightly more sensitive detector of site occupancy, but in at least three sites it remained detectable months after the target species was no longer present. eDNA-based monitoring to conserve rare species or prevent establishment of invasive species should consider how the relatively high concentration and long persistence of sedimentary eDNA can influence the spatiotemporal resolution of eDNA-based inferences. However, more research is needed before sedimentary eDNA can be routinely used to study contemporary populations. Future studies on the degradation of sedimentary eDNA and the processes moving eDNA between water and sediment would improve our understanding of how to use these reservoirs as a proxy for directly observing organisms.

5. Glossary

- **environmental DNA** (**eDNA**): DNA extracted from bulk environmental samples (e.g., soil, water, air) without isolating target organisms or their parts from the sample. eDNA can exist in multiple states along a continuum from whole living organisms (e.g., macrobial larvae or single-celled microbes) to ‘free’ extramembranous DNA molecules not bound to other particles.
- **intraorganismal eDNA**: eDNA contained in whole living organisms, such as microbes, meiofauna, or macrobial larvae, where it is protected, active, and can replicate.

- **extraorganismal eDNA**: eDNA outside of living organisms, such as cellular DNA in shed tissue, shed macrobial cells, and 'free' DNA molecules from unicellular or multicellular organisms, where it is less protected, inactive, and cannot replicate.
- **extramembranous DNA**: DNA not bound by cellular, organellar, or viral membranes; synonymous with the term extracellular DNA in microbial literature.
- **internal positive control assay** (**IPC assay**): A qPCR assay that detects and quantifies the IPC DNA.
- **internal positive control DNA** (**IPC DNA**): An exogenous DNA molecule that is spiked into a qPCR at a known and standardized concentration, then detected and quantified to determine if PCR inhibition has occurred.
- **PCR inhibition**: interference with the polymerase chain reaction caused by an excess of non-target DNA molecules, or by non-DNA substances inadvertently extracted with the DNA sample. Complete PCR inhibition causes failure to detect target DNA, and partial PCR inhibition biases quantification of target DNA.
- **quantification cycle** (**Cq**): The fractional number of qPCR thermocycles at which the reporter dye fluorescence exceeds a standardized threshold.
- **quantitative real-time polymerase chain reaction** (**qPCR**): a thermocycled chemical reaction used for targeted detection and quantification of specific nucleic acids during the reaction (in 'real time'), based on their nucleotide sequence and a fluorescent reporter dye.

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8. Figures and Tables

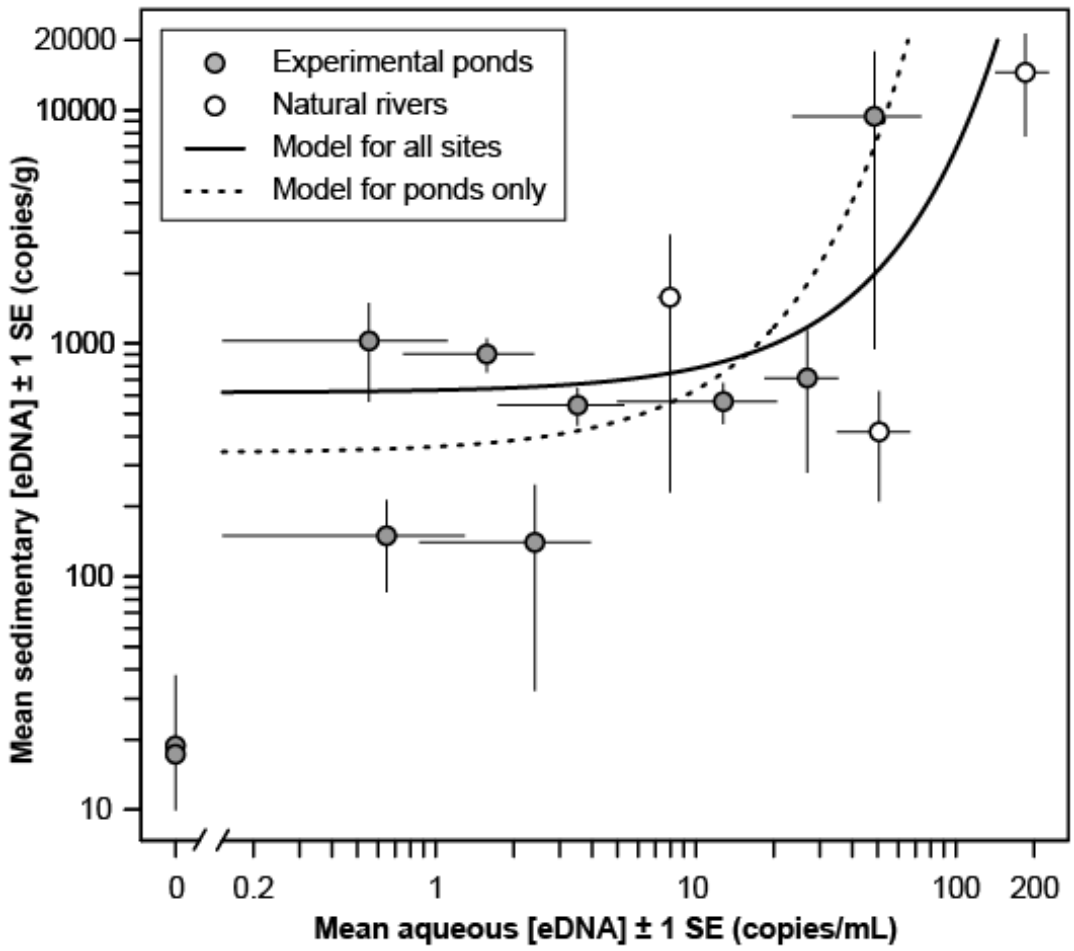

Figure 1. Plot of sedimentary and aqueous eDNA concentration for experimental ponds (n=10) and natural rivers (n=3). Filled circles show experimental ponds and unfilled circles show natural rivers. Note that both axes are logarithmic and the x-axis contains a break to include zero. The solid curve shows predicted values for the best fit GLM for all sites (P=0.01). The dashed curve shows predicted values for the best fit GLM for experimental ponds only (P=0.02).

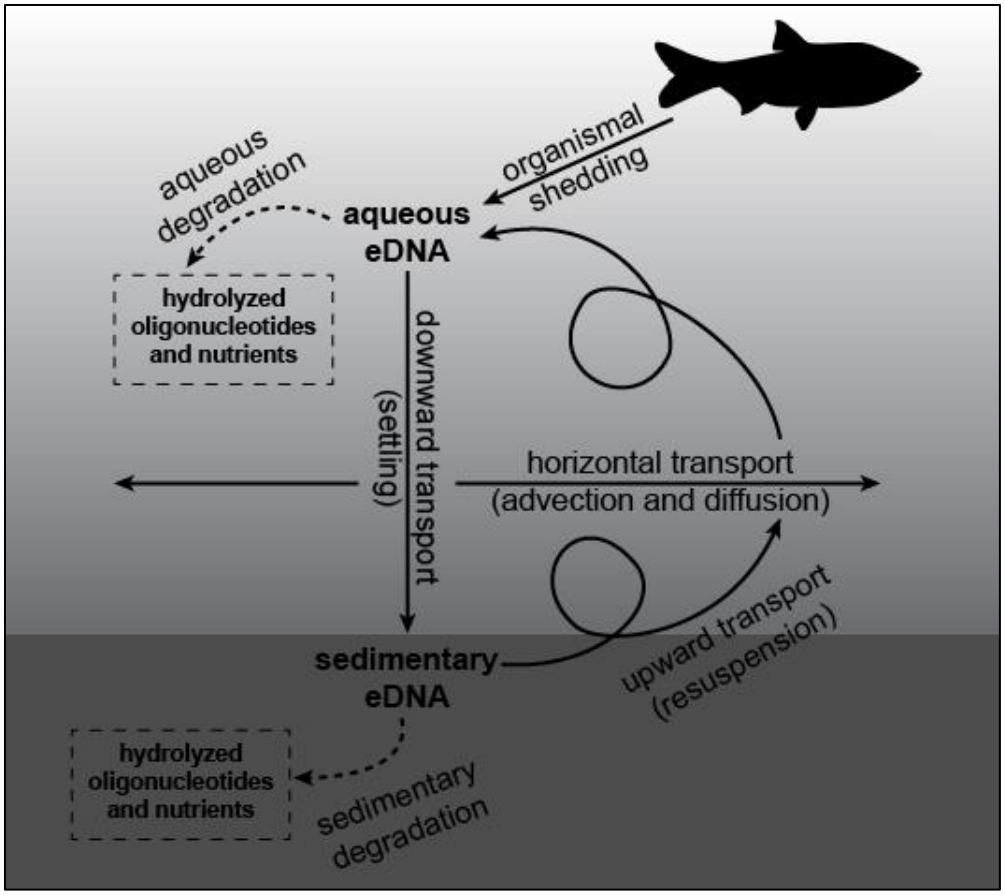

Figure 2. Conceptual diagram of the processes affecting eDNA released into the water column by aquatic macrofauna. Because sedimentary eDNA persists longer than aqueous eDNA resuspension of sediments could influence the temporal resolution of inferences about organism presence made from aqueous eDNA. Horizontal transport of resuspended sediments could also influence the spatial resolution of inferences from aqueous eDNA.

Table 1. qPCR-measured concentrations of bigheaded Asian carp (*Hypophthalmichthys* spp.) eDNA in sediment and water samples.

	Sedimentary eDNA samples					Aqueous eDNA samples					
Site	**n**	**n positive**[b]	**Mean concentration (copies g^{-1})**[c]	**SE**	**RSE**	**n**	**n positive**[b]	**Mean concentration (copies mL^{-1})**[c]	**SE**	**RSE**	**Ratio of [sedimentary eDNA] to [aqueous eDNA]**
Pond 311[a]	3	0	0	0	0	3	0	0	0	0	n/a [d]
Pond 313	3	1	19	19	100%	3	0	0	0	0	n/a [e]
Pond 316	3	3	1025	463	45%	3	1	1	1	100%	1846
Pond 317	3	2	140	108	77%	3	2	2	2	64%	58
Pond 321[a]	3	1	17	17	100%	3	0	0	0	0	n/a [e]
Pond 322	3	3	901	150	17%	3	2	2	1	52%	572
Pond 326	3	2	9425	8477	90%	3	3	49	25	52%	194
Pond 331	3	3	150	64	43%	3	1	1	1	100%	231
Pond 332	3	3	544	99	18%	3	2	4	2	51%	154
Pond 333	3	3	711	432	61%	3	3	27	8	32%	26
Pond 335	3	3	564	113	20%	3	3	13	8	61%	44
Wabash River	3	3	14 544	6829	47%	3	3	185	43	23%	79
Kansas River	3	3	418	208	50%	3	3	51	16	31%	8
Wakarusa River	2	2	1578	1348	85%	3	3	8	1	10%	198

a Pond sites were experimental ponds at the University of Kansas Field Station (KUFS) that had been stocked with at least one bigheaded carp 132-138 days before sample collection. Pond 311 was a negative control pond not stocked with fish. Pond 321 contained only one bigheaded carp for 5 days before it died and was removed (132 days before sample collection).

b A sample was positive if at least one qPCR amplified.

c Mean concentration was calculated using all samples for a site, including negative samples (zero concentration). All aqueous eDNA samples from a site were collected prior to all sedimentary eDNA samples from a site, thus no within-site pairing of water and sediment samples is possible.

d Not applicable because Pond 311 contained no fish and bigheaded carp eDNA was not detected in sediment or water.

e Ratio cannot be calculated because target eDNA was detected in sediment but not water.

n = number of eDNA samples from a site. SE = standard error of the mean. RSE = SE/mean

9. Supplementary Appendix A1.

CTAB Extraction Protocols for Sediment and Water

Extraction protocols were modified from Coyne et al. (2005, 2006, 2001).

Sediment Extraction Protocol *(steps involving Sevag should be performed inside a fume hood)*

1. Thaw the CTAB-preserved sediment sample in the fridge for no more than 24 hours.
2. Once thawed, decontaminate the exterior of the 50 mL tube with 10% bleach and rinse with reverse osmosis water.
3. Vortex at highest speed for 30 sec, then incubate at 60ºC for 10 min.
4. Add 15 ml of Sevag (Chloroform/Isoamyl alcohol 24:1).
5. Vortex the sediment/CTAB/Sevag mixture briefly and shake at low speed (Vortexer setting 4) for 5 min.
6. Centrifuge at 3220g for 15 min at room temperature to separate aqueous and organic phases.
7. Without touching the intermediate layer, carefully transfer the aqueous phase (supernatant) to a new 50 mL tube. (Tip: Use a 10 mL serological pipette for the first 8 to 12 mL, then a 1000 µL micropipette to aspirate the last 2 to 3 mL.)
8. Add an equal volume of ice cold Isopropanol and ½ volume of 5M NaCl to the supernatant and chill in a -20ºC freezer for 1 hr (or overnight if more convenient).
9. Centrifuge at 3220g for 15 min at room temperature, the carefully pour off the supernatant.
10. Add 2 mL of 70% EtOH, washing down the inner walls of the tube, then centrifuge at 3220g for 2 min at room temperature.
11. Pour off EtOH and allow the DNA pellet to air dry completely (use a 45ºC incubator to evaporate stubborn droplets).
12. Resuspend the pellet in 1000 µL of LoTE buffer. Heat briefly at 45ºC and swirl gently to mix and resuspend. Once fully resuspended, briefly centrifuge to collect all liquid in the bottom of 50 mL tube.
13. Transfer all liquid to a 1.5 µL low bind microcentrifuge tube.
14. Use 200 µL in OneStep™ Inhibitor Removal Kit (Zymo Research, Irvine, CA). This can now be used in genetic assays.

Water Extraction Protocol *(steps involving Sevag should be performed inside a fume hood)*

1. Decontaminate the exterior of the 50 mL sample tube.
2. Centrifuge the ethanol/sodium acetate-preserved water sample for 35 min at 6ºC and 3220g to precipitate and pellet eDNA and all suspended solids.
3. Pour off the supernatant and allow the pellet to air dry for ~5 min.
4. Add 700 µL of CTAB, vortex at highest speed for 30 sec, then incubate at 60ºC for 10 min.
5. Centrifuge briefly then pipette all liquid into a 2 mL microcentrifuge tube containing 700 µL of Sevag (Chloroform/Isoamyl alcohol 24:1).
6. Vortex the pellet/CTAB/Sevag mixture briefly and shake at low speed (Vortexer setting 4) for 5 min.
7. Centrifuge at 3220g for 15 min at room temperature to separate aqueous and organic phases.
8. Without touching the intermediate layer, carefully transfer the aqueous phase (supernatant) to a 1.5 µL low bind microcentrifuge tube.
9. Add an equal volume of ice cold Isopropanol and ½ volume of 5M NaCl to the supernatant and chill in a -20ºC freezer for 1 hr (or overnight if more convenient).
10. Centrifuge at 3220g for 15 min at room temperature, then carefully pour off the supernatant.
11. Add 200 µL of 70% EtOH, washing down the inner walls of the tube, then centrifuge at 3220g for 2 min at room temperature.
12. Pour off EtOH and allow the DNA pellet to air dry completely (use a 45ºC incubator to evaporate stubborn droplets).
13. Resuspend the pellet in 100 µL of LoTE buffer. Heat briefly at 45ºC and vortex gently to mix and resuspend.

CTAB Buffer Recipe

- 100mM Tris-HCL (pH 8)
- 1.4 M NaCl
- 1% (wt./vol.) Polyvinylpyrrolidone (molecular weight: 360,000, pH 8)
- 2% (wt./vol.) Cetyl trimethyl ammonium bromide (CTAB)
- 20mM EDTA (pH 8)